POST GRADUATE DEGREE PROGRAMME (CBCS) IN BOTANY

SEMESTER-IV

Course: BOTDSE T402.1

Microbiology (Course – I)

Self-LearningMaterial

DIRECTORATEOFOPENANDDISTANCELEARNING

UNIVERSITYOFKALYANIK
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Director'sMessage Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the three fold functions catered by Open and Distance Learning (ODL)systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL),University of Kalyani. Development of printed SLMs for students admitted to the DODL with in a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, NewDelhi, India under Open and Distance Mode DEB Regulations, 2020 had been our endeavour.We are happy to have achieved our goal. Utmost care and precision have been ensured in the development of the SLMs, making the useful to the learners, besides avoid in errors as far as practicable. Further suggestions from the stakeholders in this would be welcome. During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Amalendu Bhunia, Hon'ble Vice Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance. Sincere gratitude is due to the respective chairpersons as well as each and everymember of PGBOS (DODL),University of Kalyani. Heartfelt thanks are also due to the Course Writersfaculty members at the DODL, subject-experts serving at University Post Graduate departments and also to theauthorsandacademicianswhoseacademiccontributionshaveenrichedtheSLMs.We humbly acknowledge theirvaluableacademiccontributions.Iwouldespeciallyliketoconveygratitudetoallother University dignitaries and personnel involved either at the conceptual or operational level of the DODLof University of Kalyani. Their persistent and coordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode. Self-Learning Materials (SLMs) have been published by the Directorate of Open and Distance Learning, University of Kalyani, Kalyani741235, West Bengal and all the copyright reserved for University of Kalyani. No part of this work should be reproduced in any from without permission in writing from the appropriate authority of the University of Kalyani. All the Self Learning Materials are self-writing and collected frome-book, journals and websites.

Prof. Tapati Chakraborty
Director
Directorate of Open and Distance Learning
University of Kalyani

SYLLABUS
Course: BOTDSE T402.1
Microbiology (Course – I)
(Full Marks – 100)

Course	Group	Details Contents Structure		Study hour
	Microbiology (Course – I)	Unit 1. Origin and Diversity of Microorganisms:	Primitive life forms; evidence of microbial life on early earth; origin of life;	**1**
		Unit 2. Origin and Diversity of Microorganisms:	Earliest organisms and metabolic strategies.	**1**
		Unit 3. Origin and Diversity of Microorganisms:	Microbial phylogeny; universal tree of life.	**1**
		Unit 4. Origin and Diversity of Microorganisms:	Bacterial taxonomy; nomenclature and Bergey's manual	**1**
		Unit 5. Origin and Diversity of Microorganisms:	Classification and species concept	**1**
		Unit 6. Origin and Diversity of Microorganisms:	Nomenclature and formal taxonomic standing, conventional taxonomy, molecular taxonomy.	**1**
		Unit 7. Origin and Diversity of Microorganisms:	Conventional taxonomy, molecular taxonomy.	**1**
		Unit 8. Microbial Physiology and Metabolism:	Enzymes- classification and nomenclature, general properties, extraction, assay.	**1**
		Unit 9. Microbial Physiology and Metabolism:	Purification; mechanism of enzyme action, enzyme kinetics, enzyme inhibition.	**1**
		Unit 10. Microbial Physiology and Metabolism:	Carbohydrate metabolism- Embden-Meyerhoff-Parnas pathway, TCA cycle,	**1**
		Unit 11. Microbial Physiology and Metabolism:	Pentose phosphate pathway, Electron transport chain and phosphorylation.	**1**

		Unit 12. Microbial Physiology and Metabolism:	Anaerobic respiration- nitrate, sulfate, thiosulfate, elemental sulfur and carbon dioxide as electron acceptor.	**1**
		Unit 13. Microbial Physiology and Metabolism:	Fermentation- alcoholic, lactate, formate, acetate, propionate, butyrate, mixed acid and butanediol;	**1**
		Unit 14. Microbial Physiology and Metabolism:	EntnerDuodoroff pathway, Stickland reaction.	**1**
		Unit 15. Microbial Physiology and Metabolism:	Amino acid metabolism- Concept of Exo- and Endo- peptidases, transamination, deamination, transmethylation and decarboxylation;	**1**
		Unit 16. Microbial Physiology and Metabolism:	Biosynthesis of lysine, glutamic acid and phenylalanine; protein biosynthesis.	**1**
		Unit 17. Microbial Physiology and Metabolism:	Biosynthesis of phenylalanine; protein biosynthesis.	**1**
		Unit 18. Microbial Physiology and Metabolism:	Lipid metabolism- Detailed account of oxidation of saturated, unsaturated and odd carbon fatty acids.	**1**
		Unit 19. Microbial Physiology and Metabolism:	Nucleic acid metabolism- concept of purine and pyrimidine metabolism.	**1**
		Unit 20. Microbial Physiology and Metabolism:	Oxygenic and anoxygenic photosynthesis, chemosynthesis.	**1**
		Unit 21. Environmental Microbiology:	Microbial interactions- plant-microbes, animal-microbes, microbe-microbe interactions, biofilm and its significance.	**1**
		Unit 22. Environmental Microbiology:	Microbiology of air, water and soil; deep sea ecosystem-barotolerant and barophilic Bacteria.	**1**
		Unit 23. Environmental Microbiology:	Microorganisms in mineral recovery; microbial leaching of metals.	**1**
		Unit 24. Environmental Microbiology:	Biogeochemical cycling and microbes.	**1**
		Unit 25. Environmental Microbiology:	Microbes and ecological management.	**1**

	Unit 26. Environmental Microbiology:	Biomethanation from agricultural and food processing wastes.	**1**
	Unit 27. Agricultural Microbiology:	Exploitation of microbes for crop improvement and crop protection.	**1**
	Unit 28. Agricultural Microbiology:	Biological control of plant diseases and agricultural antibiotics.	**1**
	Unit 29. Agricultural Microbiology:	Biopesticides and biofertilizer.	**1**
	Unit 31. Industrial Microbiology:	Fermenters- stirred tank, bubble column, air lift, packed bed. Industrial production of ethyl alcohol	**1**
	Unit 32. Industrial Microbiology:	Industrial production of acetic acid, penicillin, vitamin B12 and amylase.	**1**

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Microbiology (Course – I)

Theoretical Course **Credits: 8**

Content Structure:

1. Introduction
2. Objectives
3. **Origin and Diversity of Microorganisms:**

• Primitive life forms; evidence of microbial life on early earth; origin of life; earliest organisms and metabolic strategies.

• Microbial phylogeny; universal tree of life.

• Bacterial taxonomy; nomenclature and Bergey's manual; classification and species concept; nomenclature and formal taxonomic standing, conventional taxonomy, molecular taxonomy.

4. **Microbial Physiology and Metabolism:**

• Enzymes- classification and nomenclature, general properties, extraction, assay and purification; mechanism of enzyme action, enzyme kinetics, enzyme inhibition.

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• Fermentation- alcoholic, lactate, formate, acetate, propionate, butyrate, mixed acid and butane diol; EntnerDuodoroff pathway, Stickland reaction.

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• Lipid metabolism- Detailed account of oxidation of saturated, unsaturated and odd carbon fatty acids.

• Nucleic acid metabolism- concept of purine and pyrimidine metabolism.

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• Industrial production of ethyl alcohol, acetic acid, penicillin, vitamin B12 and amylase.

8. Suggested reading

9. Assignment

1. Introduction

Microbiology (from Ancient Greek (mīkros) 'small', (bíos) 'life', and (-logía) 'study of') is the scientific study of microorganisms, those being of unicellular (single-celled), multicellular (consisting of complex cells), or acellular (lacking cells). Microbiology encompasses numerous sub-disciplines including virology, bacteriology, protistology, mycology, immunology, and parasitology.

Eukaryotic microorganisms possess membrane-bound organelles and include fungi and protists, whereas prokaryotic organisms—all of which are microorganisms—are conventionally classified as lacking membrane-bound organelles and include Bacteria and Archaea. Microbiologists traditionally relied on culture, staining, and microscopy for the isolation and identification of microorganisms. However, less than 1% of the microorganisms present in common environments can be cultured in isolation using current means. With the emergence of biotechnology, Microbiologists currently rely on molecular biology tools such as DNA sequence-based identification, for example, the 16S rRNA gene sequence used for bacterial identification.

Viruses have been variably classified as organisms, as they have been considered either as very simple microorganisms or very complex molecules. Prions, never considered as microorganisms, have been investigated by virologists; however, as the clinical effects traced to them were originally presumed due to chronic viral infections, virologists took a search—discovering "infectious proteins".

The existence of microorganisms was predicted many centuries before they were first observed, for example by the Jains in India and by Marcus Terentius Varro in ancient Rome. The first recorded microscope observation was of the fruiting bodies of moulds, by Robert Hooke in 1666, but the Jesuit priest Athanasius Kircher was likely the first to see microbes, which he mentioned observing in milk and putrid material in 1658. Antonie van Leeuwenhoek is considered a father of microbiology as he observed and experimented with microscopic organisms in the 1670s, using simple microscopes of his design. Scientific microbiology developed in the 19th century through the work of Louis Pasteur and in medical microbiology Robert Koch.

Here, the discipline is utilised to learn about every part of the organisms in order not only to understand how they exist in their environments but also how they affect their individual surroundings and, in turn, other organisms nearby (human beings, animals, etc.). Microbiology has consistently proved to be one of the most significant fields in biology, making it possible to define how some microorganisms cause diseases, discover treatments for such diseases and even use a few microbes for industrial applications etc.

The majority of the natural elements on the earth contain microorganisms. All living things, including humans, plants, and animals, are intimately connected to the microbes that consistently recycle important nutrients like carbon and nitrogen, break down organic matter, and influence our daily lives.

2. Course Objectives

Microbiology as special paper of M.Sc. Botany course serves to impart advanced training to the students in the field of Microbiology with focus on microbial diversity, bioprospecting and applications of microbes for obtaining various biologically significant metabolites and in bioremediation of polluted environments. Students undergo hands-on training with state-of-the art technologies and are trained so as to develop an aptitude for independent research. The Programme equips students for higher research leading to the Ph.D. Degree in India or in International Universities overseas, or for employment in Research Institutes, in teaching, and in Industry.

Learning Outcomes:

-To provide value-based education, with academic excellence and advanced research and to raise skilled candidates with research caliber in the field of Microbiology

-To inculcate the advanced concepts of Microbiology including taxonomy, physiology Immunology, biomolecular interactions, etc.

-To impart the scope for the application of concepts learned in the subject.

-To introduce about the recent advances in the field of Microbiology and its importance in research.

3. Origin and Diversity of Microorganisms:

Primitive life forms:

The **earliest known life forms** on Earth may be as old as 4.1 billion years old (or Ga) according to biologically fractionated graphite inside a single zircon grain in the Jack Hills range of Australia. The earliest evidence of life found in a stratigraphic unit, not just a single mineral grain, is the 3.7 Ga metasedimentary rocks containing graphite from the Isua Supracrustal Belt in Greenland .The earliest *direct* known life on land may be stromatolites which have been found in 3.480-billion-year-old geyserite uncovered in the Dresser Formation of the Pilbara Craton of Western Australia. Various microfossils of microorganisms have been found in 3.4 Ga rocks, including 3.465-billion-year-old Apex chert rocks from the same Australian craton region, Carbon isotopes as well as sulfur isotopes found in barite, which are fractionated by microbial metabolisms during sulfate reduction,[1] are consistent with biological processes. However, the Dresser formation was deposited in an active volcanic and hydrothermal environment, and abiotic processes could still be responsible for these fractionations. Many of these findings are supplemented by direct evidence, typically by the presence of microfossils, however.

Fossil evidence:

Fossils are direct evidence of life. In the search for the earliest life, fossils are often supplemented by geochemical evidence. The fossil record does not extend as far back as the geochemical record due to metamorphic processes that erase fossils from geologic units.

Stromatolites:

Stromatolites are laminated sedimentary structures created by photosynthetic organisms as they establish a microbial mat on a sediment surface. An important distinction for biogenicity is their convex-up structures and wavy laminations, which are typical of microbial communities who build preferentially toward the sun. A disputed report of stromatolites is from the 3.7 Ga Isua metasediments that show convex-up, conical, and domical morphologies. Further mineralogical analysis disagrees with the initial findings of internal convex-up laminae, a critical criteria for stromatolite identification, suggesting that the structures may be deformation features (i.e. boudins) caused by extensional tectonics in the Isua Supracrustal Belt.

Fig: Stromatolite fossil showing convex-up structures.

The earliest direct evidence of life are stromatolites found in 3.48 billion-year-old chert in the Dresser formation of the Pilbara Craton in Western Australia. Several features in these fossils are difficult to explain with abiotic processes, for example, the thickening of laminae over flexure crests that is expected from more sunlight. Sulfur isotopes from barite veins in the stromatolites also favor a biologic origin. However, while most scientists accept their biogenicity, abiotic explanations for these fossils cannot be fully discarded due to their hydrothermal depositional environment and debated geochemical evidence.

Most archean stromatolites older than 3.0 Ga are found in Australia or South Africa. Stratiform stromatolites from the Pilbara Craton have been identified in the 3.47 Ga Mount Ada Basalt. Barberton, South Africa hosts stratiform stromatolites in the 3.46 Hooggenoeg, 3.42 Kromberg and 3.33 Ga Mendon Formations of the Onverwacht Group. The 3.43 Ga Strelley Pool Formation in Western Australia hosts stromatolites that demonstrate vertical and horizontal changes that may demonstrate microbial communities responding to transient environmental conditions. Thus, it is likely anoxygenic or oxygenic photosynthesis has been occurring since at least 3.43 Ga Strelley Pool Formation.

Microfossils

Claims of the earliest life using fossilized microorganisms (microfossils) are from hydrothermal vent precipitates from an ancient sea-bed in the Nuvvuagittuq Belt of Quebec, Canada. These may be as old as 4.28 billion years, which would make it the oldest evidence of life on Earth, suggesting "an almost instantaneous emergence of life" after ocean

formation 4.41 billion years ago. These findings may be better explained by abiotic processes: for example, silica-rich waters, "chemical gardens," circulating hydrothermal fluids, and volcanic ejecta can produce morphologies similar to those presented in Nuvvuagittuq.

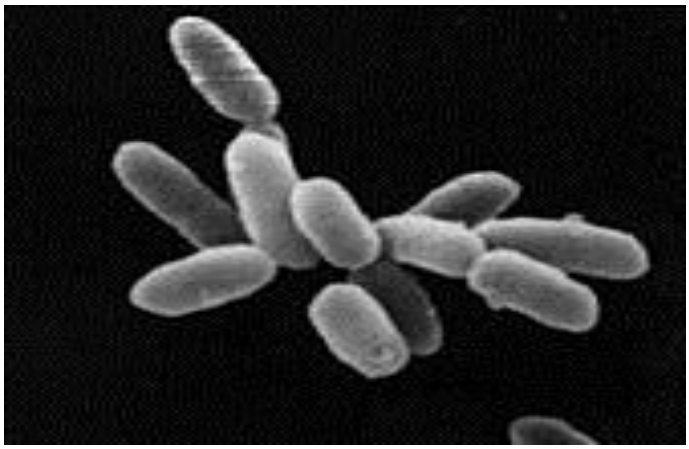


Fig : Archaea (prokaryotic microbes) were first found in extreme environments, such as hydrothermal vents.

The 3.48 Ga Dresser formation hosts microfossils of prokaryotic filaments in silica veins, the earliest fossil evidence of life on Earth, but their origins may be volcanic. 3.465-billion-year-old Australian Apex chert rocks may once have contained microorganisms, although the validity of these findings has been contested. "Putative filamentous microfossils," possibly of methanogens and/or methanotrophs that lived about 3.42-billion-year-old in "a paleo-subseafloor hydrothermal vein system of the Barberton greenstone belt, have been identified in South Africa." A diverse set of microfossil morphologies have been found in the 3.43 Ga Strelley Pool Formation including spheroid, lenticular, and film-like microstructures. Their biogenicity are strengthened by their observed chemical preservation. The early lithification of these structures allowed important chemical tracers, such as the carbon-to-nitrogen ratio, to be retained at levels higher than is typical in older, metamorphosed rock units.

The Origin of Life:

Dating meteorites through the use of radioisotopes places our planet at an estimated 4.5 to 4.6 billion years old. However, conditions on Earth for the first hundred million years or so were far too harsh to sustain any type of life. The first direct evidence of cellular life was discovered in 1977 in a geologic formation in South Africa known as the Swartkoppie chert, a granular type of silica. These microbial fossils as well as those from the Archaean Apex chert of Australia have been dated at about 3.5 billion years old . Despite these findings, the microbial fossil record is understandably sparse. Thus to piece together the very early events that led to the origin of life, biologists must rely primarily on indirect evidence. Each piece of evidence must fit together like a jigsaw puzzle for a coherent picture to emerge.

Microbial Diversity:

In this section, we examine the wide diversity of microbial life. We shall also consider some specific examples, particularly with respect to their effect on humans. By way of introduction, however, we need to say something on the subject of the classification of microorganisms. In any discussion on biological classification, it is impossible to avoid mentioning Linnaeus, the Swedish botanist who attempted to bring order to the naming of living things by giving each type a Latin name. He even gave himself one – his real name was Carl von Linn´e! It was Linnaeus who was responsible for introducing the binomial system of nomenclature, by which each organism was assigned a genus and a species. To give a few familiar examples, you and I are Homo sapiens, the fruit fly that has contributed so much to our understanding of genetics is Drosophila melanogaster, and, in the microbial world, the bacterium responsible for causing anthrax is Bacillus anthracis. Note the following conventions, which apply to the naming of all living things (the naming of viruses is something of a special case:1. The generic (genus) name is always given a capital letter 2. The specific (species) name is given a small letter The generic and specific name are italicised, or, if this isn't possible, underlined 3. The science of taxonomy involves not just naming organisms,but grouping them with other organisms thatshare common properties. In the early days, classification appeared relatively straightforward, with all livingthings apparently fitting into one of two kingdoms. Tooversimplify the matter, if it ran around, it was an animal,if it was green and didn't, it was a plant! As ourawareness of the microbial world developed, however, itwas clear that such a scheme was not satisfactory to accommodate all life forms, and inthe mid-19th century, Ernst Haeckel proposed a third kingdom, the Protista, to includethe bacteria, fungi, protozoans and algae. In the 20th

century, an increased focus on the cellular and molecular similaritiesand dissimilarities between organisms led to proposals for further refinements to thethree-kingdom system. One of the most widely accepted of these has been the five kingdomsystem proposed by Robert Whittaker in 1969 . Like some of itspredecessors, this took into account the fundamental difference in cell structure betweenprokaryotes and eukaryotes, and so placed prokaryotes (bacteria) in theirown kingdom, the Monera, separate from single-celled eukaryotes. Another feature ofWhittaker's scheme was to assign the Fungi to their own kingdom, largely on account oftheir distinctive mode of nutrition. Table A1 shows some of the characteristic featuresof each kingdom. Molecular studies in the 1970s revealed that the

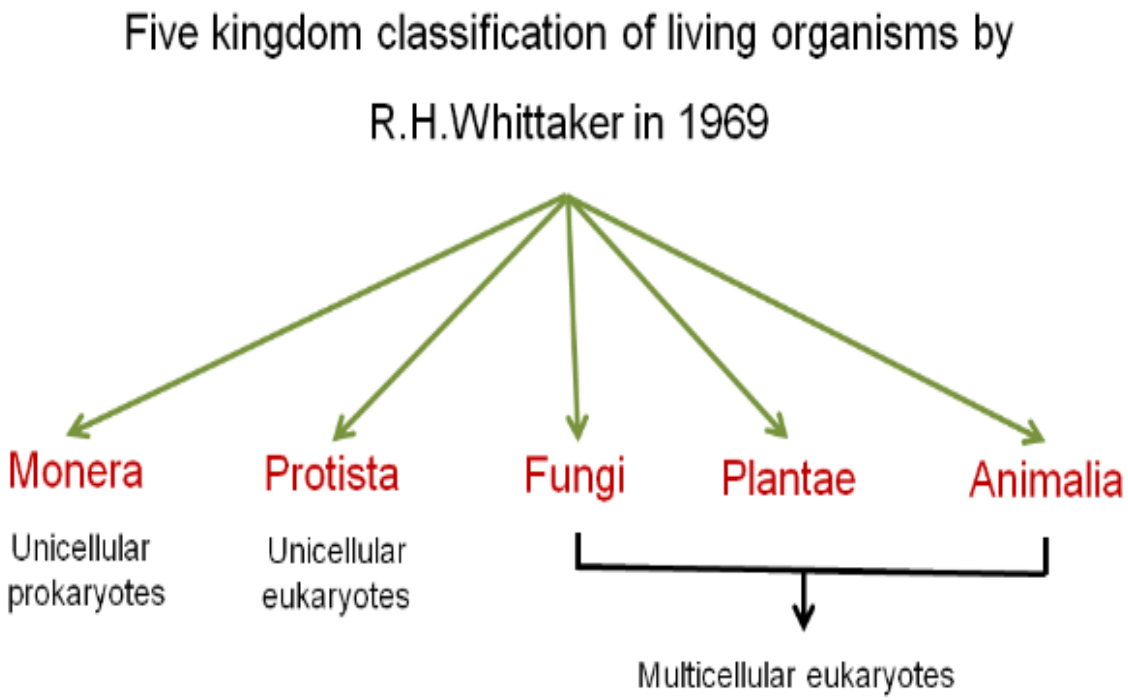


Figure 3. Archaea differed from all other bacteriain their 16S rRNA sequences, as well as in their cell wall structure, membrane lipidsand aspects of protein synthesis. These differences were seen as sufficiently important for the recognition of a third basic cell type to add to the prokaryotes and eukaryotes.

This led to the proposal of a three-domain scheme of classification, in which prokaryotes are divided into the Archaea and the Bacteria. The third domain, the Eucarya represents all eukaryotic organisms. The domains thus represent a level of classification that goes even higher than the kingdoms. Although the Archaea (the word means 'ancient') represent a more primitive bacterial form than the Bacteria, they are in certain respects more closely related to the eukaryotes, causing biologists to revise their ideas about the evolution of the eukaryotic state. In hierarchical systems of classification, related species are grouped together in the same genus, genera sharing common features are placed in the same family, and so on. Table A2 shows a modern classification scheme for the gut bacterium Escherichia coli. The boundaries between longstanding divisions such as algae and protozoa have become considerably blurred in recent years, and alternative classifications based on molecular data have been proposed. This is very much a developing field, and no definitive alternative classification has yet gained universal acceptance. of the word, that is, unicellular eukaryotic forms. It retains the traditional distinction between protozoans, algae and other protists (water moulds and slime moulds), but also offers an alternative, 'molecular' scheme, showing the putative phylogenetic relationship between the various groups of organisms. Microbiology has traditionally embraced anomalies such as the giant seaweeds, as it has encompassed all organisms that fall outside of the plant and animal kingdoms. This book offers only a brief consideration of such macroscopic forms, and for the most part confines itself to the truly microbial world. The viruses, it ought to be clear by now, are special cases. Because an understanding of viruses requires an appreciation of the basics of DNA replication and protein synthesis.

Domain:

Archaea Studies on 16S ribosomal RNA sequences by Carl Woese and colleagues allowed the construction of phylogenetic trees for the prokaryotes, showing their evolutionary relatedness. . The work of Woese also revealed that one group of prokaryotes differed from all the others. Archaea are now regarded as being quite distinct from the Bacteria (sometimes called Eubacteria). Together with the Eucarya, these form the three domains of life. Extending nucleic acid analysis to other genes has shown that members of the Archaea possess many genes not found in any other type of bacteria.

General features of the Archaea

Members of the Archaea show considerable diversity of both morphology and physiology. In view of the fact that the Archaea remained unidentified as a separate group for so many years, it should come as no surprise that they do not display any obvious morphological differences from true bacteria, and all the main cell shapes are rep- resented. More unusual shapes are also encountered in archaea; members of the genus Haloarcula have flattened square or triangular cells! Both Gram-positive and Gram- negative forms of archaea are found, but neither possesses true peptidoglycan. Some types have a so-called pseudomurein, composed of different substituted polysaccharides and L-amino acids . Most archaea, however, have cell walls composed of a layer of proteinaceous subunits known as an S-layer, directly associated with the cell membrane. This difference in cell wall chemistry means that members of the Archaea are not susceptible to antibacterial agents such as lysozyme and penicillin, whose action is directed specifically towards peptidoglycan. Differences are also found in the make-upof Archaean membranes, where the lipid component of membranes contains branched isoprenes instead of fatty acids, and these are joined to glycerol by ether-linkages, rather than the ester-linkages found in true bacteria. The diversity of archaea ex- tends into their adopted means of nutrition and metabolism: aerobic/anaerobic and autotrophic/heterotrophic forms are known. Many members of the Archaea are found in extreme environments such as deep-sea thermal vents and salt ponds. Some extreme thermophiles are able to grow at temperatures well over 100 ◦C, while psychrophilic forms constitute a substantial proportion of the microbial population of Antarctica. Similarly, examples are to be found of archaea that are active at extremes of acidity, alkalinity or salinity. Initially it was felt that archaea were limited to such environments because there they faced little competition from true bacteria or eucaryotes. Recent stud- ies have shown however that archaea are more widespread in their distribution, making up a significant proportion of the bacterial biomass found in the world's oceans, and also being found in terrestrial and semiterrestrial niches. The reason that this lay unde-tected for so long is that these organisms cannot as yet be cultured in the laboratory, and their presence can only be inferred by the use of modern DNA-based analysis.

Domain: Bacteria All the remaining bacterial groups belong to the domain Bacteria. This is divided into 23 phyla the more important of which are discussed in the following pages, according to their description in the second edition of Bergey. As with the Archaea, many other forms are known only through molecular analysis and it is estimated that these represent at least another 20 phyla.

Phylum: Proteobacteria: We start our survey of the Bacteria with the Proteobacteria. This is by far the biggest single phylum, and occupies the whole of volume 2 in the second edition of Bergey. The size of the group is matched by its diversity, both morphological and physiological; most forms of metabolism are represented, and the wide range of morphological forms gives rise to the group's name. (Proteus was a mythological Greek god who was able to assume many different forms.) The reason such a diverse range of organismshave been assigned to a single taxonomic grouping is that their 16S rRNA indicates a common ancestor (thought to be photosynthetic, though few members now retain this ability). At the time of writing more than 460 genera and 1600 species had been identified, all of them Gram-negative and representing almost half of all accepted bacterial genera. These include many of the best known Gram-negative bacteria of medical, industrial and agricultural importance. For taxonomic purposes, the Proteobacteria 18 have been divided into five classes reflecting their presumed lines of descent and termed the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria andEpsilonproteobacteria(Figure7.4).Itshouldbestressedthatbecauseclassification is based on molecular relatedness rather than shared phenotypic traits, few if any mor- phological or physiological properties can be said to be characteristic of all members of each class. Equally, organisms united by a particular feature may be found in more than one of the proteobacterial classes, for example nitrifying bacteria are to be found in the α, β and γ Protobacteria. For this reason, in the following paragraphs we describe the Proteobacteria interms of their phenotypi ccharacteristics ratherthan attempt to group them phylogenetically.

Microbial phylogeny: Phylogenetic relationships are illustrated in the form of branched diagrams or trees. A phylogenetic tree is a graph made of branches that connect nodes. The nodes represent taxonomic units such as species or genes; the external nodes at the end of the branches represent living (extant) organisms. As in the universal phylogenetic tree, the length of the branches represents the number of molecular changes that have taken place

between the two nodes. Finally, a tree may be unrooted or rooted. An unrooted tree simply represents phylogenetic relationships but does not provide an evolutionary path. Figure 19.13a shows that Ais more closely related to C than it is to either B or D, but does not specify the common ancestor for the four species or the direction of change. In contrast, the rooted tree gives a node that serves as the common ancestor and shows the development of the four species from this root. It is much more difficult to develop a rooted tree. For example, there are 15 possible rooted trees that connect four species, but only three possible unrooted trees. Phylogenetic trees are developed by comparing nucleotide or amino acid sequences. To compare two molecules, their sequences must first be aligned so that similar parts match up. The object is to align and compare homologous sequences, ones that are similar because they had a common origin in the past. This is not an easy task, and computers and fairly complex mathematics must be employed to minimize the number of gaps and mismatches in the sequences being compared. Once the molecules have been aligned, the numbers of positions that vary in the sequences are determined. These data are used to calculate a measure of the difference between the sequences. Often the difference is expressed as the evolutionarydistance. This is simply a quantitative indication of the number of positions that differ between two aligned macromolecules. Statistical adjustments are made for back mutations and multiple substitutions that may have occurred. Organisms are then clustered together based on similarity in the sequences. The most similar organisms are clustered together, then compared with the remaining organisms to form a larger cluster associated together at a lower level of similarity or evolutionary distance. The process continues until all organisms are included in the tree. Phylogenetic relationships also can be estimated by techniques such as parsimony analysis. In this approach, relationships are determined by estimating the minimum number of sequence changes required to give the final sequences being compared. It is presumed that evolutionary change occurs along the shortest pathway with the fewest changes or steps from an ancestor to the organism in question. The tree or pattern of relationships is favored that is simplest and requires the fewest assumptions.

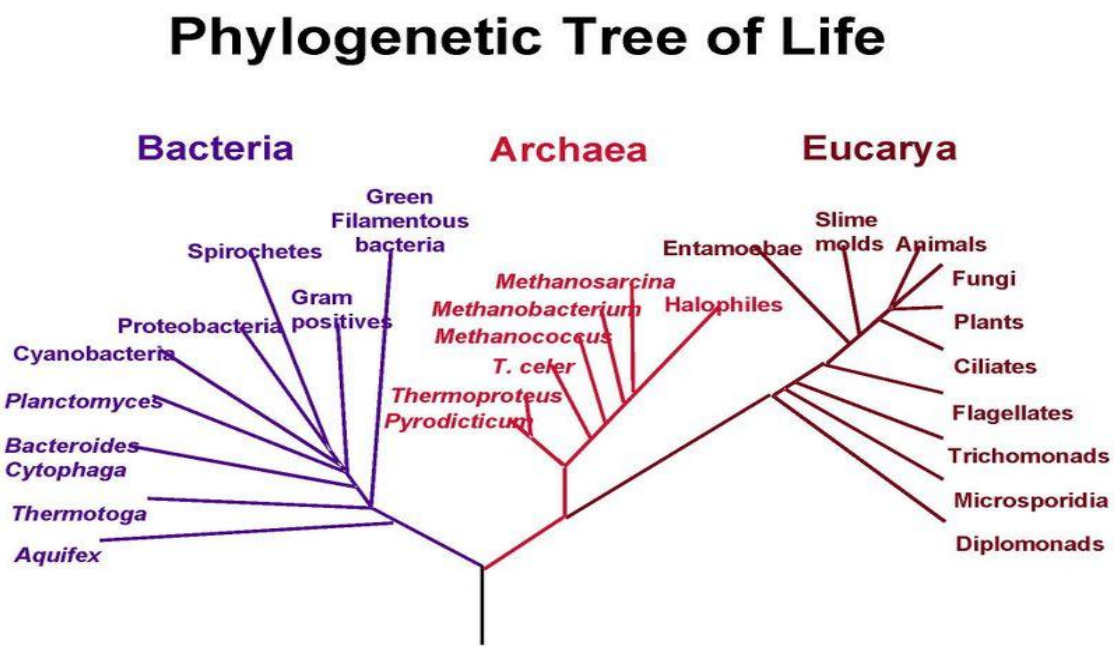


Fig: Phylogenetic tree of life

Methods for Microbial Phylogenetic Analysis

Most microbial taxa have never been cultivated or experimentally characterized. Utilizing taxonomy and phylogeny are essential tools for organizing the diversity of life. Collecting gene sequences, aligning such sequences based on homologies and thus using models of mutation to infer evolutionary history are common methods to estimate microbial phylogenies. Small subunit (SSU) rRNA (SSU rRNA) have revolutionized microbial classification since the 1970s and has since become the most sequenced gene. Phylogenetic inferences are determined based on the genes chosen, for example, 16S rRNA gene is commonly selected to investigate inferences in Bacteria and Archaea, and microbial eukaryotes most commonly use the 18S RNA gene.

Phylogenetic comparative methods

Phylogenetic comparative methods (PCMs) are commonly utilized to compare multiple traits across organisms. Within the scope of microbiome studies, it is not common for the use of PCMs, however, recent studies have been successful in identifying genes associated with colonization of human gut. This challenge was addressed through measuring the statistical association between a species that harbors the gene and the probability the species is present in the gut microbiome. The analyses showcase the combination of shotgun metagenomics paired with phylogenetically aware models.

Ancestral state reconstruction

This method is commonly used for estimation of genetic and metabolic profiles of extant communities using a set of reference genomes, commonly performed with PICRUSt (Phylogenetic Investigation of Communities by Reconstructing of Unobserved States) in microbiome studies. PICRUSt is a computational approach capable of prediction functional composition of a metagenome with marker data and a database of reference genomes. To predict which gene families are present, PICRUSt uses extended ancestral-state reconstruction algorithm and then combines the gene families to estimate composite metagenome.

Analysis of phylogenetic variables and distances

Phylogenetic variables are used to describe variables that are constructed using features in the phylogeny to summarize and contrast data of species in the phylogenetic tree. Microbiome datasets can be simplifies using phylogenetic variables by reducing the dimensions of the data to a few variables carrying biological information. Recent methods such as PhILR and phylofactorization address the challenges of phylogenetic variables analysis. The PhILR transform combines statistical and phylogenetic models to overcome compositional data challenges. Incorporating both microbial evolutionary models with the isometric log-ratio transform creates the PhILR transform. Phylofactorization is a dimensionality-reducing tool used to identify edges in the phylogeny from which putative functional ecological traits may have arisen.

Challenges

Inferences in phylogenetics requires the assumption of common ancestry or homology but when this assumption is violated the signal can be disrupted by noise. It is possible for microbial traits to be unrelated due to horizontal gene transfer causing the taxonomic composition to reveal little about the function of a system.

Importance of Phylogeny in Microbiology

Prokaryotic organisms evolved around 3 billion years ago, representing a time that included the majority of early evolutionary life history. Studies of prokaryotic evolutionary relationships are important for understanding the origin and diversification of life on earth.

Phylogeny combined with taxonomy creates a universal language for understanding what an organism is and where it fits in the broad tree of life. Such information can be used to identify emerging pathogens and develop new methods of treating infections.

Early microbial phylogeny

The reliable grouping of microorganisms and their relationships to each other is a fundamental process for all later microbiological research. Early studies arranged prokaryotic organisms into groups based on morphological or biochemical characteristics.

Analyses between the 18th and mid-20th century were unable to produce a definitive classification system for microorganisms that reflected their genealogies based on these characteristics.

The advent of molecular analysis advanced phylogenetic studies, particularly the sequence characteristics of small subunit ribosomal RNA (SSU rRNA).

The existence of both highly conserved and variable regions of the molecule, along with its universal presence, makes small subunit ribosomal RNA an important tool for determining the evolutionary relationship between organisms.

This method of producing phylogenetic connections has been used as the primary basis for classifying prokaryotic organisms and phylogenetic trees show that the majority of biodiversity is microbial.

Phylogenetic analysis has been employed to study microbial communities in a range of ecosystems including ocean depths and in hosts such as the human microbiome.

Limitations of SSU rRNA phylogenetics

Studies employing small subunit ribosomal RNA as a phylogenetic marker are limited because of their reliance on cultivated microorganisms and the utilization of a single gene to define similarities and differences.

The relatively short sequences of less than 500 nucleotides in length often used in phylogenetic analysis represent only one-third of the total length of 16S rRNA. Many scientists believe that this number of nucleotides provides insufficient comparative information for an accurate phylogenetic tree.

Evolutionary distant SSU rRNA genes with similar nucleotide composition have consistently been placed close together in phylogenetic trees, a clear indication of a method that is not robust. Such studies rely on corroboration from other phylogenetic markers to assess the accuracy of the phylogenetic tree produced.

Metagenomes and phylogeny

Metagenomic data, the collective genetic material derived directly from an environmental sample, can be used to study the phylogenetic context of microbial diversity. The method can sample a wide range of genes at once.

A phylogenetic reconstruction is formed from homology with sequenced genomes of isolated strains.

The decreasing cost of sequencing technologies and the improving ability to sequence complete microbial genomes promises to enhance taxonomic classification and the phylogenetic placement of metagenomic data, produced from multiple gene families.

The development of reference databases will also aid the phylogenetic assignment of metagenomic data as more genomes are sequenced.

Metagenomic data is currently being used to revolutionize research into human intestinal microbes. This example highlights the importance of phylogeny to microbiology for identifying activities performed by an evolutionary line of species.

Studies of the human colonic microbiota have previously highlighted exclusive methane production to a small number of archaeal lineages. The linking of functionality to phylogeny has important implications for the identification of therapeutic targets when function correlates to host health.

Correct identification of species is therefore critical for the correct choice of antibiotics or for producing novel employment as probiotics.

Methods and Programs

The purpose of phylogenetic analysis is to understand the past evolutionary path of organisms. Even though we will never know for certain the true phylogeny of any organism, phylogenetic analysis provides best assumptions, thereby providing a framework for various disciplines in microbiology. Due to the technological innovation of modern molecular biology and the rapid advancement in computational science, accurate inference of the phylogeny of a gene or organism seems possible in the near future. There has been a flood of nucleic acid sequence information, bioinformatic tools and phylogenetic inference methods in public domain databases, literature and worldwide web space. Phylogenetic analysis has long played a central role in basic microbiology, for example in taxonomy and ecology. In addition, more recently emerging fields of microbiology, including comparative genomics and phylogenomics, require substantial knowledge and understanding of phylogenetic analysis and computational skills to handle the large-scale data involved. Methods of phylogenetic analysis and relevant computer software tools lend accuracy, efficiency and availability to the task.

There are four steps in general phylogenetic analysis of molecular sequences: (i) selection of a suitable molecule or molecules (phylogenetic marker), (ii) acquisition of molecular sequences, (iii) multiple sequence alignment (MSA) and (iv) phylogenetic treeing and evaluation. The first step of phylogenetic analysis is to choose a suitable homologous part of the genomes to be compared. Mechanisms of molecular evolution include mutations, duplication of genes, reorganization of genomes, and genetic exchanges such as recombination, reassortment and lateral gene transfer. Although all of this information can be used to infer phylogenetic relationships of genes or organisms, information on mutations, including substitution, insertion, and deletion, is most frequently used in phylogeny reconstruction. The aim is to infer a correct organismal phylogeny, using orthologous genetic loci, in which common ancestry of two sequences can be traced back to a speciation event. Phylogeny using homologous genetic loci derived by gene duplication (paralogy) or related through lateral gene transfer (xenology), cannot reflect evolutionary history of organisms.

Once DNA sequence data are generated, they are subjected to a multiple sequence alignment process. This involves finding homologous sites, that is, positions derived from the same ancestral organism in the molecules under study. A set of sequences can be aligned with another by introducing "alignment gaps" (known in brief as "gaps"). In general, multiple sequence alignment starts by aligning a pair of sequences (pairwise alignment), and is then expanded to multiple sequences using various algorithms.

Many algorithms and computer programs have been developed in the last few decades for multiple sequence alignment, but the original Clustal series programs are still most widely used and produce reasonably good quality MSA for small data sets. For a large dataset, such as massive pyrosequencing reads, the MUSCLE program can generate good compromise between accuracy and speed. The MAFFT program utilizes several different algorithmic approaches and can be used for either small or very large datasets. There are also other computer programs developed for general multiple sequence alignment, but the above three have been most popular and are routinely used in publications in various microbiological disciplines read more.

Multilocus Sequence Analysis

Multilocus sequence analysis (MLSA) represents the novel standard in microbial molecular systematics. In this context, MLSA is implemented in a relatively straightforward way, consisting essentially in the concatenation of several sequence partitions for the same set of

organisms, resulting in a "supermatrix" which is used to infer a phylogeny by means of distance-matrix or optimality criterion-based methods. This approach is expected to have an increased resolving power due to the large number of characters analyzed, and a lower sensitivity to the impact of conflicting signals (i.e. phylogenetic incongruence) that result from eventual horizontal gene transfer events. The strategies used to deal with multiple partitions can be grouped in three broad categories: the total evidence, separate analysis and combination approaches. The concatenation approach that dominates MLSAs in the microbial molecular systematics literature is known to systematists working with plants and animals as the "total molecular evidence" approach, and has been used to solve difficult phylogenetic questions such as the relationships among the major groups of cetaceans, that of microsporidia and fungi, or the phylogeny of major plant lineages. The total molecular evidence approach has been criticized because by directly concatenating all available sequence alignments, the evidence of conflicting phylogenetic signals in the different data partitions is lost along with the possibility to uncover the evolutionary processes that gave rise to such contradictory signals. The nature of these conflicts is varied, but in the microbial world the strongest conflicting signals often derive from the existence of horizontal gene transfer events in the dataset. If the individuals containing xenologous loci are not identified and removed from the supermatrix prior to phylogeny inference, the resulting hypothesis may be strongly distorted, since standard treeing methods assume a single underlying evolutionary history. Based on these arguments, the conditional data combination strategy is to be generally preferred in bacterial MLSA read more.

rRNA and Other Global Markers

The introduction of comparative rRNA sequence analysis represents a major milestone in the history of microbiology. The current taxonomy of prokaryotes as well as modern probe and chip based identification methods are mainly based upon rRNA derived phylogenetic conclusions. Also of importance is single gene based phylogenetic inference and alternative global markers include elongation and initiation factors, RNA polymerase subunits, DNA gyrases, heat shock and recA proteins. Although the comparative analyses are hampered by the generally low phylogenetic information content, and different resolution power, and multiple copies of the individual markers, the domain and prokaryotic phyla concept is globally supported read more.

The Phyla of Prokaryotes

There is no official classification of prokaryotes. For the higher taxa there even is no official

nomenclature: the rules of the International Code of Nomenclature of Prokaryotes do not cover taxa above the rank of class. The most commonly accepted division of the prokaryotes in two "subkingdoms" or "domains" (Bacteria and Archaea) and the classification of their species with validly published names in respectively 27 and 2 "phyla" or "divisions" (as of November 2009) is primarily based on 16S rRNA sequence comparisons. This type of classification was adopted in the latest edition of Bergey's Manual of Systematic Bacteriology. Alternative classifications have been proposed as well, based e.g. on the structure of the cell wall. Some 16S rRNA sequence-based phyla unite prokaryotes of similar physiological properties (for example Cyanobacteria, Chlorobi, Thermotogae); others (Euryarchaeota, Proteobacteria, Flavobacteria) contain organisms with highly disparate lifestyles. Some phyla based on deep 16S rRNA lineages are currently represented by one or a few species only. Environmental genomics/metagenomics approaches suggest existence of many more phyla based on the deep lineages of 16S rRNA gene sequences recovered. To obtain the organisms harboring these sequences and to study their properties is a major challenge of microbiology today read more.

Rooting the Tree of Life

Defining the evolutionary relationships between groups of organisms is a major part of modern-day microbiology. With the continuing dramatic increase in the availability of genomic data, these techniques have been extended to describing an all-encompassing "tree of life". However, identifying the location of the root of this tree corresponding to the most recent common ancestor is a challenging and distinct problem that has yet to be solved. To date, many investigations have proposed various roots, using a wide diversity of biological data and techniques. A survey of the most promising of these models illustrates the difficulty faced in reaching a scientific consensus on the issue, as well as the additional philosophical complications posed by our emerging understanding of the role of horizontal gene transfer in genome evolution read more ...

Conserved Indels

Comparative analysis of genome sequences is leading to discovery of large numbers of novel molecular markers that are proving very helpful in understanding many important aspects of microbial phylogeny. Of these molecular markers, the conserved inserts or deletions (indels) in protein sequences provide particularly useful means for identifying different groups of microbes in clear molecular terms and for understanding how they have branched off from a common ancestor. Conserved indels and other novel molecular markers

(viz. lineage-specific proteins) can be useful for understanding microbial phylogeny at different phylogenetic depths. Genetic and biochemical studies of these markers should also lead to identification of novel properties that are unique to different groups of microbes read more.

Lateral or Horizontal Gene Transfer

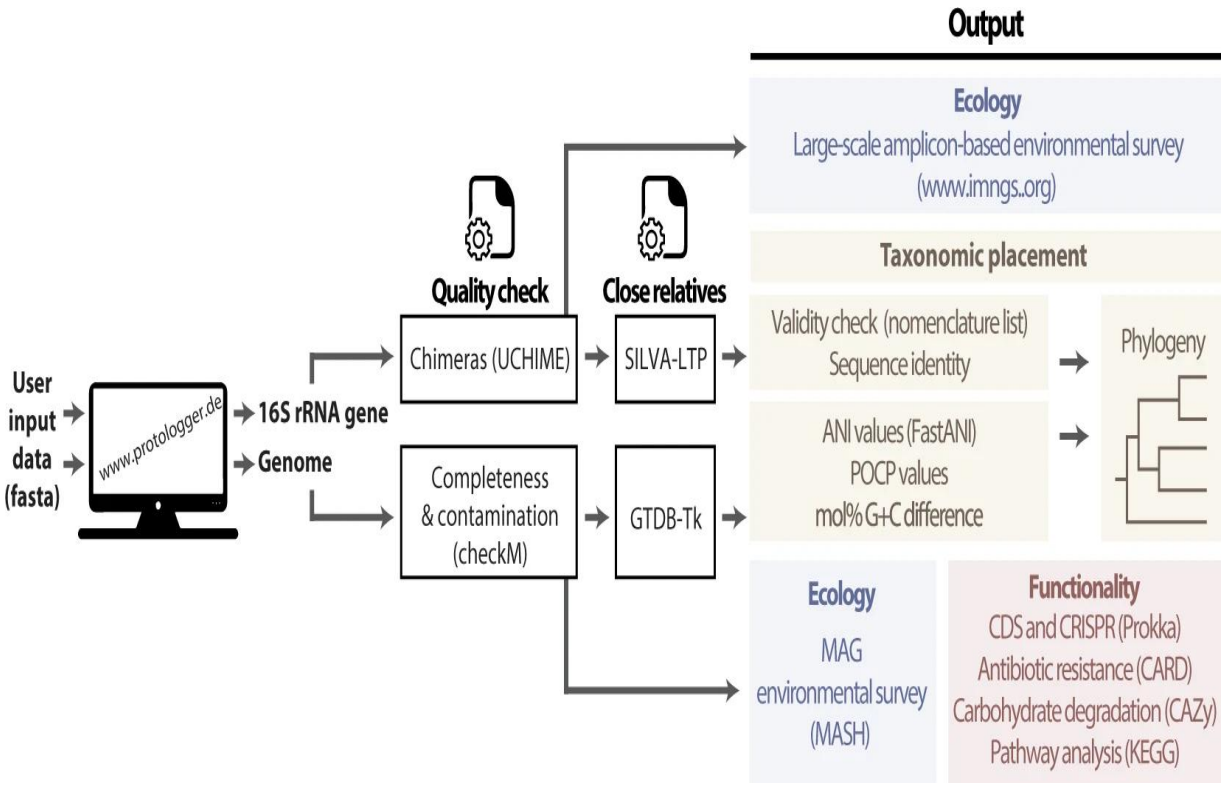
Efforts to construct the tree of life take their conceptual motivation from Charles Darwin's theory of evolution. Until the advent of molecular biology, however, a universal tree of life was well beyond the scope of the data and methods of traditional organismal phylogeny. The rapid development of these methods and bodies of genetic sequence from the 1970s onwards resulted in major reclassifications of life and revived ambitions to represent all organismal lineages by one true tree of life. Subsequent realization of the significance of lateral gene transfer and other non-vertical processes has subtly reconceptualized and reoriented attempts to construct this universal phylogeny.

Gene transfer has affected the formation of groups of organisms. Gene transfer can make it more difficult to define and determine relationships. In those cases where many genes have been transferred between preferred partners, the majority of genes in a genome may reflect gene acquisition, and as a consequence, if a coherent signal is detected, one nevertheless might not be sure that the signal is due to organismal shared ancestry. However, the presence of a particular transferred gene has been shown, in several cases, to constitute a shared derived character useful in classification. Gene transfer can put together new metabolic pathways that open up new ecological niches, and consequently, the transfer of an adaptive gene might create a new group of organisms read more.

Endosymbiosis and the Evolution of Plastids

Photosynthesis is one of the most successful energy production strategies on the planet and has been co-opted numerous times throughout evolutionary history via the uptake and retention of photosynthetic cells by non-photosynthetic eukaryotic heterotrophs. Whereas the result of this process is clear, what is not settled is the mode and tempo of plastid movement among eukaryotes, particularly plastids of red algal derivation. Recent changes in our understanding of the relationships between eukaryotic supergroups have only served to complicate the picture further.

Of particular interest is the evolution of plastids, the relationships among photosynthetic eukaryotes, the process of endosymbiogenesis and the variation in ways plastids have been modified to suit the light harvesting needs of their hosts. The understanding of all of these factors is an active field of continued research that will undoubtedly lead to further discoveries in the coming years read more.



The key steps within Protologger are highlighted with the tools utilised for each step indicated (in brackets), along with the quality assurance steps. Sections are coloured according to the information they provide with taxonomic placement (in yellow), ecology (in blue), and functionality (in red). The ‘validity check’ stage in taxonomic assignment involves the removal of taxa without validly published names from genomic comparison.

Bacterial taxonomy: The art of biological classification is known as "Taxonomy". It consists of 3 important parameters viz. Classification, Identification & Nomenclature. It is a branch of Biology which deals with systematic classification of living organism. Generally, 2 types of classifications are distinguished- Artificial & Natural. The artificial system serves mainly arrange living organism in a way so that they may be correctly identified. The natural system on the other hand strikes to extend the scope beyond mere identification rather this system aims to arrange living organism according to their relatedness. Besides these two, there is another system of classification which mainly deals with evolutionary relatedness. It is obvious that evolutionary relatedness between any two relative groups is proportional to the no. of genes common between them. Such system of classification is known as Phylogenetic system of Classification.

1. **Classical Taxonomy of Bacteria**: In classification, it is generally used as many characters as possible to determine genetic similarity with higher plants and animals. Morphological and sometimes physiological characteristics, developmental homology and fossil records more or less satisfactory to build up phylogenetic or natural system of classification. The same principle cannot be applied with equal success in case of Bacteria. Bacteria generally have only limited variation in morphology. They have a great spectrum of variation in physiological & biochemical characteristic. But the later characteristics are offered subject to alteration under the influence of environment. Hence, they are not very often suitable for classification. Nor to the bacteria show any ontogenic development or any reliable fossil record. For the lack of reliable stable criteria for classification, the systems so far develop for bacteria are mainly artificial. The system is, however, may useful to identify unknown freshly isolated bacteria and relating them to previously described species. The properties which are used to identify the bacteria include- microscopic characteristics such as Shape, Size, Motility, Flagellation, Endospore, Capsule, Stainability etc. ; microscopic morphology of the colonies like Colour, Shape, Margin, Elevation, Soluble pigment etc.. Apart from these a large no. of physiolological properties and Biochemical characteristics are also used such as Carbon (C) Nitrogen (N) source utilization, pH & temperature, Oxygen relationship etc. Serological properties are also be used for bacterial identification. Following morphological, cultural and physio-biochemical charaters are used in phenetic classification.

2. **Molecular Taxonomy of Bacteria:** Modern approach of bacterial taxonomy has undergone drastic changes after the development of molecular biology under the 2nd half of 20th century. The concept that the macromolecules like proteins, lipids could be used as indicator of the organism was first suggested by Zuckerkandl & Pauling in 1965.

i) They described as the macromolecules are used as molecular characteristic.

ii) The sequence of monomers in them changed slowly and randomly.

iii) The no. of changes in the particular macromolecule as increased linearly with geological time scale.

iv) The comparison of sequence of monomers of particular macromolecules from two organisms should give a measure for their phylogenetic relatedness.

v) Initially amino acid sequence of a particular protein is used as parameters; but it is now replaced by Nucleic acids. Among the nucleic acids following characters are used in molecular taxonomya) DNA-based composition. b) Nucleic Acid Hybridization (DNA-DNA). c) Ribosomal DNA homology (rDNA homology). d) Multilocus sequence typing / Multilocus sequence alignment. e) Whole genome analysis.

▪ **Tm**=69.3+0.41(G+C) % G+C % of DNA can also be determined by buoyant density of ds DNA. ▪ Buoyant density (p)=1.660+0.98(G+C)% Advantages of GC content:

1) The variation in the percentage of GC content is not more than 3% within the well-defined species.

2) The variation not more than 10% GC content within the well-defined genus. GC content data are taxonomically important in 2 ways a) They can confirm a taxonomic scheme developed by other data. If organisms in the same taxon are too dissimilar in GC percentage the taxon should be divided. b) It is useful to characterize the genus since the variation within a genus is generally less than 10%.

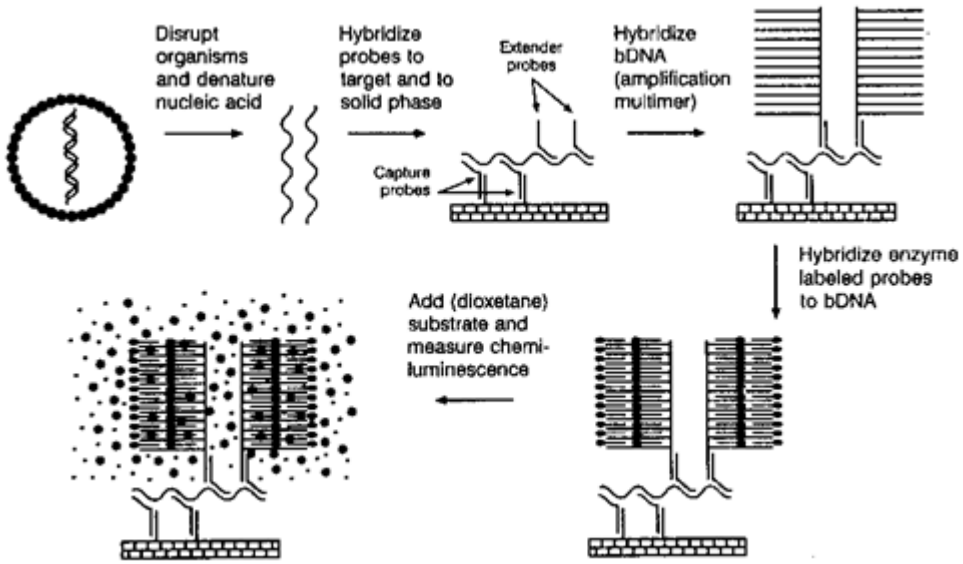
➢ Disadvantages of GC content:

1) It is not safe to assume that the organism having similar GC% ,they are also similar. The GC% is taxonomically misleading.

2) GC content gives only the overall composition of DNA and not gives any information about the sequence of the DNA bases.

3) So, GC content of the DNA does not give any information regarding the dissimilarity of the genes of the two organisms. So, GC content although a good taxonomic characteristic but cannot be used for bacterial identification rather it can be used carefully along with other characteristics of the organism.

Nucleic acid hybridization: Hybridization is a basic property of nucleotide sequences and is taken advantage of in numerous molecular biology techniques. Overall, genetic relatedness of two species can be determined by hybridizing segments of their DNA (DNA-DNA hybridization). Due to sequence similarity between closely related organisms, higher temperatures are required to melt such DNA hybrids when compared to more distantly related organisms. A variety of different methods use hybridization to pinpoint the origin of a DNA sample, including the polymerase chain reaction (PCR). In another technique, short DNA sequences are hybridized to cellular mRNAs to identify expressed genes. Pharmaceutical drug companies are exploring the use of antisense RNA to bind to undesired mRNA, preventing the ribosome from translating the mRNA into protein. Hybridization is a basic property of nucleotide sequences and is taken advantage of in numerous molecular biology techniques. Overall, genetic relatedness of two species can be determined by hybridizing segments of their DNA (DNA-DNA hybridization). Due to sequence similarity between closely related organisms, higher temperatures are required to melt such DNA hybrids when compared to more distantly related organisms. A variety of different methods use hybridization to pinpoint the origin of a DNA sample, including the polymerase chain reaction (PCR). In another technique, short DNA sequences are hybridized to cellular mRNAs to identify expressed genes. Pharmaceutical drug companies are exploring the use of antisense RNA to bind to undesired mRNA, preventing the ribosome from translating the mRNA into protein.



Advantage of Nucleic Acid Hybridization: 1) It gives a quantitative measure of degree of the complementary sequence of two DNA from two different bacterial isolate i.e. homology between two DNA. 2) 70% DNA and their **Tm value have not more than 5% differences belongs to the same species. 3) It can solve many problems in bacterial taxonomy particularly at species level.**

➢ **Disadvantage of Nucleic Acid Hybridization:** 1) For determining the relationship among distantly related organism, it cannot give any positive information. Only information can be obtained which is related or not related to each other. But, it has no value in taxonomic point of view. 2) It has high experimental error. 3) In accurate reproducibility of the result failure to generate cumulative database.

c) Ribosomal DNA (rDNA) homology: Various genes are used in molecular phylogenetic studies, among which 16s rRNA (SSU=Small SubUnit) genes have been extensively used for sequence based evolutionary analysis.

This can be used because –

i) universally distributed in bacteria,

ii) functionally constant,

iii) sufficiently conserved (slowly changed in evolution),

iv) adequate in length (5s rRNA = 123bp, 23s rRNA = 3300bp, 16s rRNA=1650bp),

v) v) can provide a view of evolution. 5s rRNA is too short to provide required information, 23s rRNA is too long to handle, but 16s rRNA has a reasonable length. Carl Woese, the University of Illianois, worked on SSU rRNA for phylogenetic studies. His work established that the presence of 3 domains of life viz. namely Archaea, Eukarya & Bacteria.

SSU rRNA gene sequence has employed in— **i) Signature sequence, ii) Phylogenetic probe & FISH, iii) Microbial Community Analysis, iv) Ribotyping, v) Sequence homology.**

i) **Signature sequence:** a) Ribosomal RNA of most of the taxonomic group possesses 1 or more unique sequences which are known as Oligonucleotide signature or signature sequence. b)Such signature defining a specific group within a domain or particular genus or single species. c) Because of their exclusivity it is very useful for newly isolated bacteria. d)Previously misclassified organism may be corrected into distinct phylogenetic group. This sequence used to design a specific nucleic acid probe.

Signature sequences in proteins could be defined as regions in the alignments where a specific change is observed in the primary structure of a protein in all members of one or more taxa but not in the other taxa. The changes in the sequence could be either the presence of particular amino acid substitutions or specific deletions or insertions (i.e., indels). In all cases, the signatures must be flanked by regions that are conserved in all the sequences under consideration. These conserved regions serve as anchors to ensure that the observed signature is not an artifact resulting from improper alignment or from sequencing errors.

The rationale of using conserved indels in evolutionary studies could briefly be described as follows. When a conserved indel of defined length and sequence, and flanked by conserved regions (which ensure that the observed changes are not due to improper alignment or sequencing errors), is found at precisely the same position in homologs from different species, the simplest and most parsimonious explanation for this observation is that the indel was introduced only once during the course of evolution and then passed on to all descendants. This is a minimal assumption implicit in most evolutionary analyses. Thus, based on the presence or absence of a signature sequence, the species containing or lacking the signature can be divided into two distinct groups,

which bear a specific evolutionary relationship to each other. A well-defined indel in a gene or protein also provides a very useful milestone for evolutionary events, since all species emerging from the ancestral cell in which the indel was first introduced are expected to contain the indel whereas all species that existed before this event or which did not evolve from this ancestor will lack the indel. Further, if specific indels could be identified in proteins that coincide with or were introduced at critical branch points during the course of evolution, such signatures could serve as important phylogenetic markers for distinguishing among major groups of organisms.

In using conserved indels as phylogenetic markers, two potentially serious problems that could affect the interpretation of any data should be kept in mind. **First**, there is the possibility that the observed indel was introduced on multiple occasions in different species due to similar functional constraints and selection pressure rather than being derived from a common ancestor. **Second**, lateral gene transfer between species could also readily account for the presence of shared sequence features in particular groups of organisms. While a definitive resolution of the question whether a given sequence signature is due to common ancestry or results from these two causes is difficult in most cases, important insights concerning the significance of such data are often provided by consideration of information from other sources.

The most important and relevant information bearing on this issue is provided by consideration of cell structure and physiology. In this context, it should be emphasized that the aim of phylogenetic analysis is to explain and reconstruct the evolutionary history of organisms. Hence, the structural and physiological characteristics of organisms are of central importance, and they should be the ultimate arbiter in determining the significance of such data. Without this context, phylogenetic analysis of sequence data could become an end in itself, bearing little relation to the organisms. Therefore, if the inference derived from a given signature sequence or phylogenetic analysis is consistent with an important structural (e.g., cell envelope structure) or physiological attribute of the organisms, it is likely that we are on the right track, and it gives confidence in the correctness of the inference.

On the other hand, if the inferences based on signature sequences and phylogenetic analyses are at a variance with important structural and physiological characteristics, one should ask questions about why it is so rather than distrusting or ignoring these characteristics.

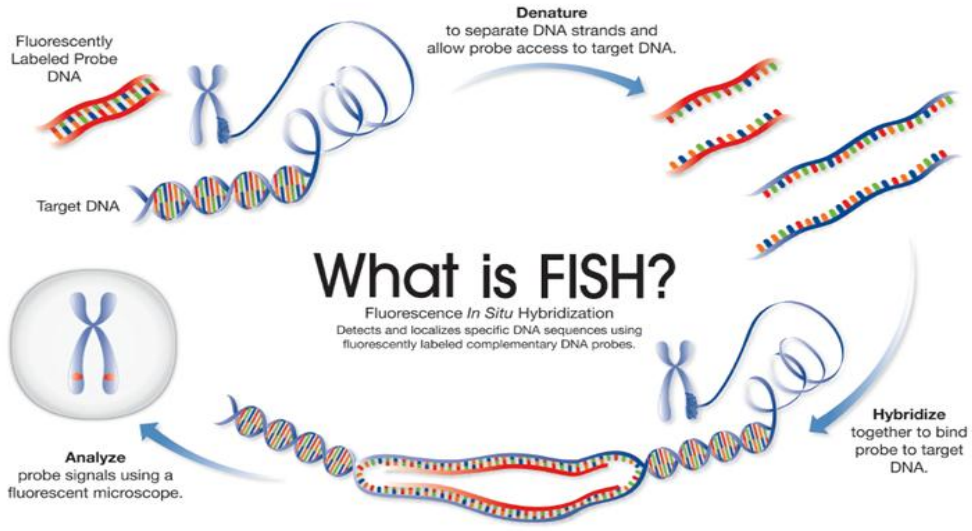
Another useful criterion in assessing whether a given signature is of evolutionary significance is provided by its species distribution. If a given sequence signature is present in all known members of a given taxa, it is more probable that it was introduced only once in a common ancestor of the group and then passed on to all descendants. In such cases, phylogenies based on other gene sequences are also expected to be generally consistent with and support the inference drawn from the signature. In contrast, when a shared indel is present either in only certain members of particular taxa or when species containing the signature show no obvious structural or physiological relationship, the possibility that the observed signature is a result of independent evolutionary events or horizontal gene transfers becomes more likely. In our analysis, we have come across several examples of signature sequences which provide evidence of lateral gene transfers between species (unpublished results).

The presence of well-defined signature sequences in proteins should allow one to establish evolutionary relationships among species by means of molecular cladistic analysis. This approach, although not generally applicable to all proteins (because most proteins do not contain useful sequence signatures), has certain advantages over traditional phylogenetic analyses based on the gene or protein sequences. First, in traditional phylogenetic analysis, the evolutionary relationships among different species are determined based upon the assumption of a constancy of evolutionary rate in all species. Since this assumption is rarely correct over long periods, the differences in evolutionary rates could lead to incorrect species relationships. However, the signature sequences, such as conserved indels of defined sizes, should not be greatly affected by the differences in evolutionary rates.

The proteins which are greatly affected by the differences in evolutionary rates are unlikely to contain well-defined indels in conserved regions and hence will be excluded from consideration. A second common and serious source of problems in phylogenetic analysis involves sequencing errors, and anyone involved in DNA sequencing should be familiar with this. For example, sequence compressions which are not satisfactorily resolved are a common occurrence, particularly in G+C-rich sequences. The errors introduced in reading such regions could lead to either localized (from base and amino acid substitutions) or extended (from frameshifts) changes in the gene or protein sequences. In one study, the error frequency in DNA sequences in the databases has been estimated at 3.55%, although other estimates indicate it to be much lower. An additional but related problem involves the increasing number of sequences in the databases which have been obtained by PCR amplification and sequenced by automated means. The higher rates of sequence errors and contamination in such sequences should be a cause of concern. These factors could affect the branching orders of species in phylogenetic trees. However, it is highly unlikely that a sequencing error could give rise to an indel of a defined length and sequence at a precise position within a conserved region. A signature of even one amino acid involves the addition or deletion of three nucleotides in the DNA sequence at a precise position and hence is highly significant. Third, a very common problem in evolutionary analyses (discussed in the previous section) is that the phylogenetic trees based on certain genes (or proteins) may fail to resolve the branching orders (e.g., low bootstrap scores for the nodes) for particular groups of species and hence the results of these studies will be indeterminate; i.e., they neither support nor refute a particular relationship. However, this is not a problem in the case of signature sequences, where the relationship is assessed based on the presence or absence of a given signature and thus its interpretation is unambiguous. One expects that the relationship indicated by signature sequences should generally be consistent with and supported by the phylogenetic analysis based on other gene or protein sequences.

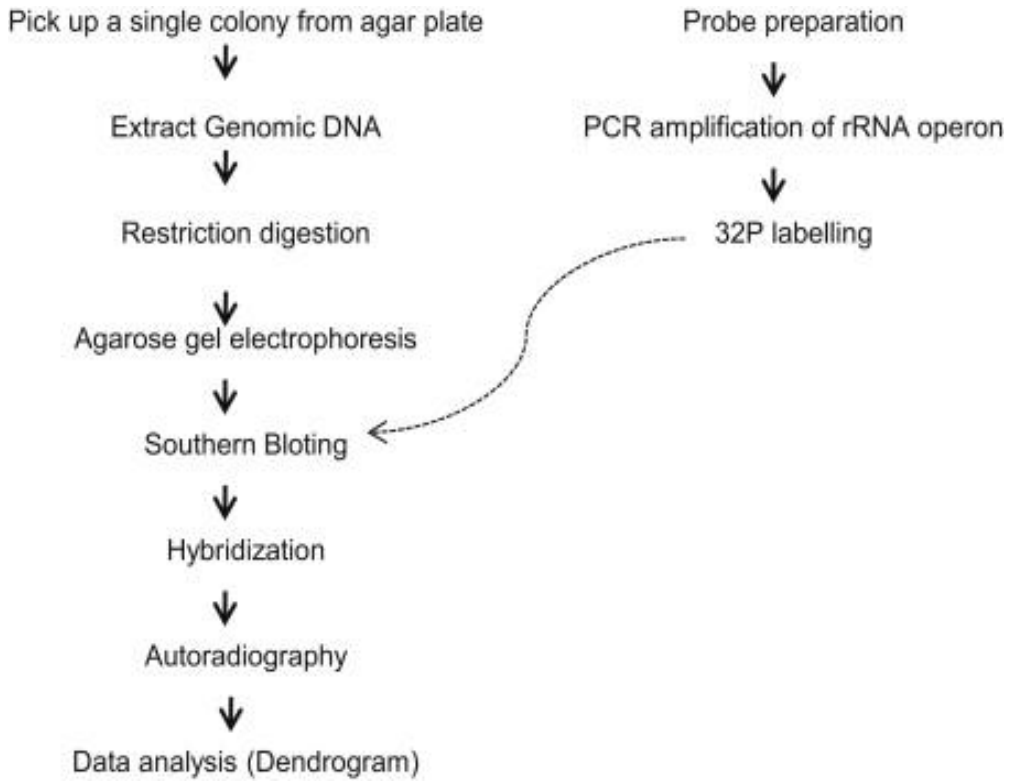
However, the analyses based on signature sequences are limited in one sense: whereas a phylogenetic tree provides information about evolutionary interrelationships among all species in a tree, a given signature sequence is limited to distinguishing and establishing the evolutionary relationship between the two groups of species, i.e., those containing and those lacking the signature.

ii) **Phylogenetic probe & FISH:** a) A probe is a strand of nucleic acid that can be labeled and used to hybridize a complementary nucleic acid from a mixture. b) Universally 16S rRNA probes are designed to bind the conserved sequence in the ribosomal RNA of all bacteria and that will react only with specific unique signature sequence. c) This is known as Phylogenetic probe and it can be designed also to target groups within a domain such as members of the individual families, genus or species. d) Binding of the probe to cellular ribosome can be seen microscopically when the fluorescent dye is attached to the probe. e) By treating with appropriate reagent membrane becomes permeable and allowed to penetrate the probe with dye mixture. f) After hybridization of the probe directly binds to the rRNA in ribosomes can be observed under Fluorescent Microscope. This technique is known as FISH (Fluorescent in situ Hybridization). g) In essence FISH is a phylogenetic stain. FISH technology is widely used in Microbial Ecology and clinical diagnosis.



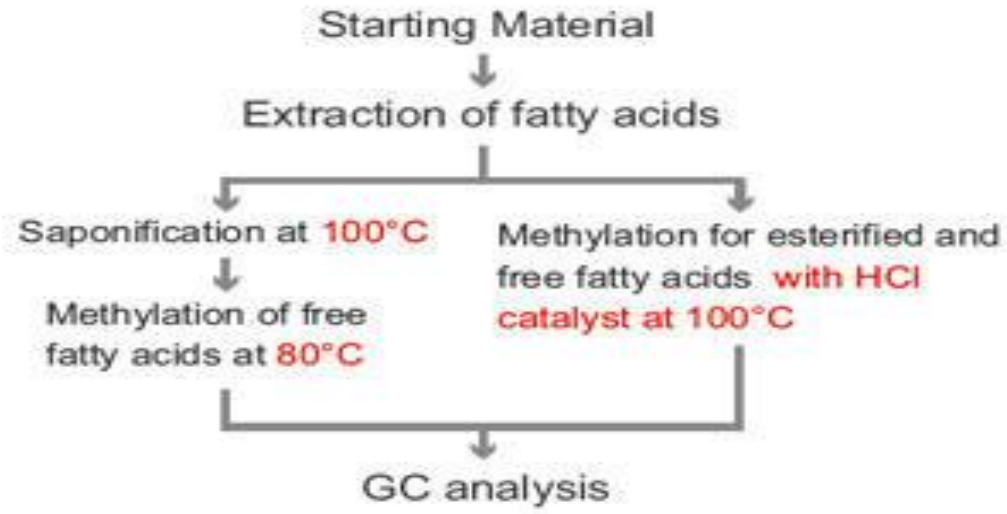
iii) **Microbial Community Analysis:** The rRNA or rDNA homology was studied in 1997 with pure culture of bacteria. The technique has been developed to recover rRNA gene directly from natural habitats. This eliminates the necessity to grow bacteria in laboratory pure culture. Phylogenetic snapshots can be taken using PCR amplified rRNA genes encoding SSU rRNA from members of that community. Such genes can be easily sequenced and aligned. A phylogenetic tree can be generated from the environmental sequences that sequences that shows the differences from the rRNA community. From this phylogenetic tree, a specific organism can be inferred even more than actually cultivated or identified. Such microbial community analysis is a major thrust of microbial ecological research today.

iv) **Ribotyping:** Information from rRNA based phylogenetic analysis also find application in a technique for bacterial identification called ribotyping. Unlike the comparative sequencing method ribotyping does not involve sequencing. Instead, it gives specific patterns of bands or DNA fingerprints i.e. generated when the DNA from an organism is digested by Restriction enzymes & the fragments are separated and probed with ribosomal RNA gene probe. Hence the size and numbers of band detected and generates specific pattern, a kind of genome finger printing called ribotype. This pattern can be compared with patterns of reference organisms in a computer. Difference between organisms in the sequence of their 16S S rRNA gene translate in the presence or absence of cutting sites recognized by different restriction enzymes. The DNA banding pattern or ribotype of a particular bacterial species may be unique and diagnostic allowing discrimination between species and different strains of each species if there are differences in their 16S rRNA gene sequence.



v) ❖**Chemotaxonomy:** FAME (Fatty Acid Methyl Ester) Test: Fatty acid is present in the cytoplasmic membrane and outer membrane of gram negative bacteria. The techniques for determination of those fatty acids have been called as FAME. Widely used in clinical, public health, food and water inspection laboratories where the identification of the pathogen and other bacterial hazards needs to be done. It is widely used in characterization of new species of bacteria. The fatty acid composition of bacteria is highly variable in bacteria including difference in chain length, presence or absence of double bonds, rings or branch rings, chain rings or hydroxyl groups etc. Hence, fatty acid profile can often be used to identify a particular bacterial species. For the analysis fatty acid are extracted from the cell in laboratory by standardized methods. Then the fatty acids are chemically derivatized to form corresponding methyl ester. These new volatile derivatives are then identified by gas chromatography. A chromatogram showing the types and amounts of fatty acids from unknown bacterium is then compared with the database containing, the fatty acid profile of thousands of the reference bacteria grown under same

condition.



Bargey's Manual: There was need for a single practical scheme that could cover all the described bacteria. From 1916 to 1918 Robert Buchanan was the first to prepare such a comprehensive scheme in a series of paper. In 1917, the society of American Bacteriology now called as American Society of Microbiology (ASM)] appointed a committee to coordinate all these information and the final report of all these committee came in 1920s based largely on Buchanan's work provided the written document and the beginning of new outline of bacterial classification. During this period David H. Bargey began preparing a more complete review of enormous literature of bacterial taxonomy. To aid the publication of this work The Society of American Bacteriologists appointed as bacterial board with Dr. Bargey. The other members of board were Harrison, Breed, Hamer and Frank. The first edition of the Bargey's manual was published in 1923. The board with change in membership and David Bargey is continuing as chairman brought out a 2nd edition of the manual in 1925 and 3rd edition in 1930. Dr.Bargey while preparing the 4th edition in 1934, requested The Society of American Bacteriologist to make availability the royalties received by society from the cell of earlier editions to meet the expenses of preparing the 4th edition for publication. Such publication was made by the society, but the use of society's financial machinery proved a burden to the society as well as the editorial board. Consequently an agreement between the society and Dr.Bargey was made that the society would transfer to Dr.Bargey all its rights, titles and interests in the manual and Dr.Bargey would in turn create an educational trust to which all the rights would be transferred.

Hence, Dr. Bargey would become the nominal owner of the manual. On 2nd Jan, 1936, he executed a trust where the members were Dr.Bagey, Breed and Murray as the initial trusty and transferred to the trusty to their successor, the ownership of the manual. The trust is a non-profit organization and its copyright and the rights to receive income earn from its publication. The trust is till now has published successively the 4th, 5th, 6th, 7th, 8th and 9th edition of the manual in 1934, 1939, 1948, 1957, 1974 and 1994 respectively. These are The Bargey's Manual Determinative Bacteriology. The trust in 1977 also published in abbreviated version of 8 th edition entitled The Shorter Bargey's Manual of Deteminative Bacteriology. Until 1974, the manual was perceived largely as an American classification of Bacteria, although, it was used in other countries as well. In 1974, the manual began to become truly international with cooperative effort. Authorities from all over the world invited to prepare the description of various genera and species. So, the 8th edition of manual contains contribution of 135 authors of the world. In 1984, another major change occurs, the scope of manual was greatly broaden to bring together information dealing with ecology, enrichment, isolation, preservation and characterization of bacteria all of which concern bacterial classification and identification. The new breadth of coverage was reflected by new name "Bargey's Manual of Systematic Bacteriology". The new edition of this work prepare with 4 volumes and with contribution of 100s of microbiologists. Standard publication of 9th edition of Bargey's Manual Determinative Bacteriology in 1994 was published by William and Wilkins, Baltimore, USA. The 9th edition is intended solely for identification of Bacteria that have described. The arrangement of the book is strictly phenotypic and no attempt has been made to offer a natural classification. The arrangement chosen is arbitrary and intended to aid in identification of bacteria. The publication divides bacteria into 35 groups. The 2 nd edition of Bargey's Manual Systematic Bacteriology in where Garrity G.M. is a chief editor and published it in 5 Volumes. It has been published by Springer Verlag of Newyork, USA in collaboration with Bargey's Manual trust. In the 5th volume bacteria are arranged phylogenetically in which comparison of nucleic acid sequences particularly the 16s rRNA gene sequences has given importance for the foundation of new classification.

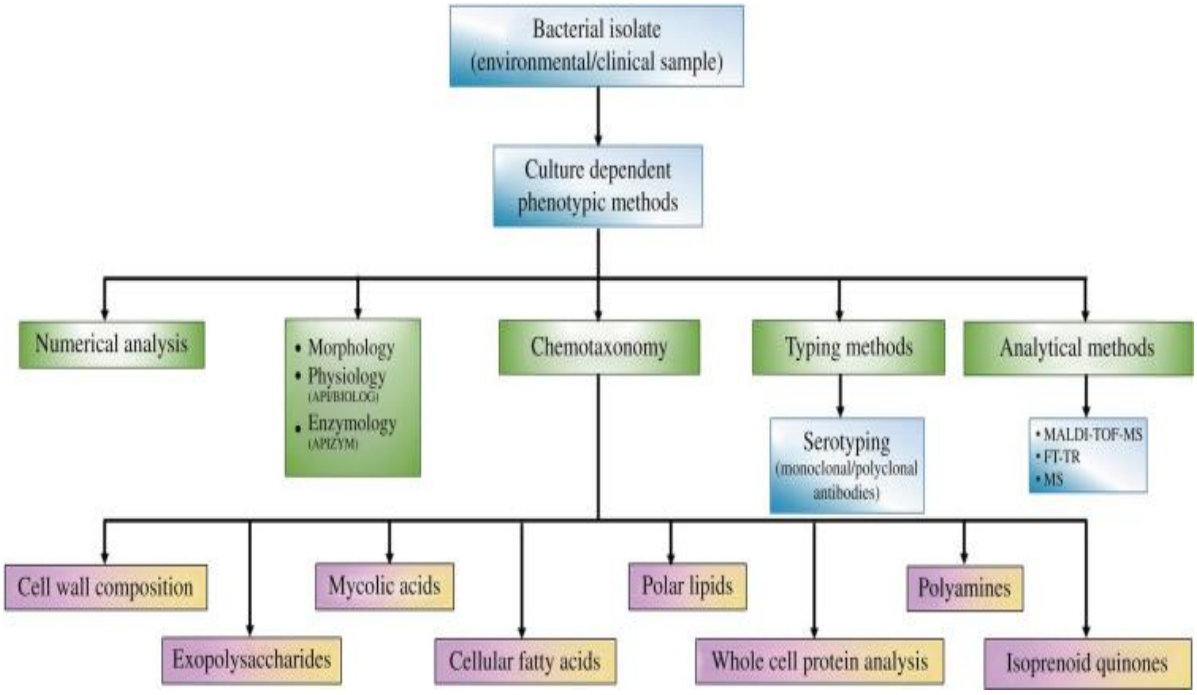
Bergey's Manual of Determinative Bacteriology (1923-1994) ▪ 9 th Editions (1 volume each)-These are mainly phenetic 2. Bergey's Manual of Systematic Bacteriology: ▪ 1 s t edition (4volumes);1984-1989; Mix Phylogenetic/Phenetic-5 Kingdoms ▪ 2 nd Edition (5 volumes) (2001-2012);Phylogenetic-3 Domains 3. Bergey's Manual of Systematic

BacteriologyFirst edition -Published in 4 volumes: Volume 1 (1984) -Gram-negative Bacteria of general, medical, or industrial importance Volume 2 (1986) -Gram-positive Bacteria other than Actinomycetes Volume 3 (1989) -Archaeobacteria, Cyanobacteria, and remaining Gram-negative Bacteria Volume 4 (1989) -Actinomycetes 4. 2 nd Edition of Bergey's Manual of Systematic Bacteriology published in following 5 volumes:- Vol 1-(2001) The Archaea and the deeply branching and phototrophic Bacteria Vol 2-(2005)-The Proteobacteria Vol 3-(2009)- The Low G+C Gram-Positive Bacteria Vol 4-(2011)- The High G+C Gram Positive bacteria Vol 5-(2012)- The Planctomycetes, Spirochaetes, Fibrobacteres, Bacteroidetes and Fusobacteria

The Species Concept in Microbiology ▪ No universally accepted concept of species for prokaryotes ▪ Current definition of prokaryotic species ▪ Collection of strains sharing a high degree of similarity in several independent traits ▪ Most important traits include 70% or greater DNA-DNA hybridization and 97% or greater 16S rRNA gene sequence identity

Polyphasic Taxonomy: Over the last 25 years, a much broader range of taxonomic studies of bacteria has gradually replaced the former reliance upon morphological, physiological, and biochemical characterization. This polyphasic taxonomy takes into account all available phenotypic and genotypic data and integrates them in a consensus type of classification, framed in a general phylogeny derived from 16S rRNA sequence analysis. In some cases, the consensus classification is a compromise containing a minimum of contradictions. It is thought that the more parameters that will become available in the future, the more polyphasic classification will gain stability. In this review, the practice of polyphasic taxonomy is discussed for four groups of bacteria chosen for their relevance, complexity, or both: the genera Xanthomonas and Campylobacter, the lactic acid bacteria, and the family Comamonadaceae. An evaluation of our present insights, the conclusions derived from it, and the perspectives of polyphasic taxonomy are discussed, emphasizing the keystone role of the species. Taxonomists did not succeed in standardizing species delimitation by using percent DNA hybridization values. Together with the absence of another "gold standard" for species definition, this has an enormous repercussion on bacterial taxonomy. This problem is faced in polyphasic taxonomy, which does not depend on a theory, a hypothesis, or a set of rules, presenting a pragmatic approach to a consensus type of taxonomy, integrating all available data maximally. In the future, polyphasic taxonomy will have to cope with (i) enormous amounts of data, (ii) large numbers of strains, and (iii) data fusion (data

aggregation), which will demand efficient and centralized data storage. In the future, taxonomic studies will require collaborative efforts by specialized laboratories even more than now is the case. Whether these future developments will guarantee a more stable consensus classification remains an open question.



A Polyphasic Taxonomic Approach for Designation and Description of Novel Microbial Species

COLLECTION CENTRE OF TYPE CULTURE:

Most Microbiological laboratories usually maintain a large collection of pure culture as well as subculture of the authentic species. The culture maintained in viable condition under particular laboratory condition, known as Stock Culture Collection. The stock culture collection generally retains the entire characteristics initially described stock culture collection centre which has been established throughout the world to help microbiologists.

Such stock culture collection is also known as Type Culture Collection from which anyone can take microbes for their use. The name and address of some culture collection centre are given below--- 1. American Type Culture Collection Centre, 12301 Park Lawn Drive, Rocky Villae, Maryland, USA. 2. Institute Pasteur, Paris, France. 3. USSR Antibiotic Research Institute in Muscow, USSR. 4. Microbial Type Culture Collection Centre (MTCC), Institute of Microbial Technology, Chandigarh, India. 5. Microbial Culture Collection Centre (MCC), Pune.

4. Microbial Physiology and Metabolism:

Enzymes: According to the International Union of Biochemists (I U B), enzymes are divided into six functional classes and are classified based on the type of reaction in which they are used to catalyze. The six kinds of enzymes are hydrolases, oxidoreductases, lyases, transferases, ligases and isomerases.

Listed below is the classification of enzymes discussed in detail:

Types	Biochemical Property
Oxidoreductases	The enzyme Oxidoreductase catalyzes the oxidation reaction where the electrons tend to travel from one form of a molecule to the other.
Transferases	The Transferases enzymes help in the transportation of the functional group among acceptors and donor molecules.
Hydrolases	Hydrolases are hydrolytic enzymes, which catalyze the hydrolysis reaction by adding water to cleave the bond and hydrolyze it.
Lyases	Adds water, carbon dioxide or ammonia across double bonds or eliminate these to create double bonds.
Isomerases	The Isomerases enzymes catalyze the structural shifts present in a molecule, thus causing the change in the shape of the molecule.
Ligases	The Ligases enzymes are known to charge the catalysis of a ligation process.

Oxidoreductases

These catalyze oxidation and reduction reactions, e.g. pyruvate dehydrogenase, catalysing the oxidation of pyruvate to acetyl coenzyme A.

Transferases

These catalyze transferring of the chemical group from one to another compound. An example is a transaminase, which transfers an amino group from one molecule to another.

Hydrolases

They catalyze the hydrolysis of a bond. For example, the enzyme pepsin hydrolyzes peptide bonds in proteins.

Lyases

These catalyze the breakage of bonds without catalysis, e.g. aldolase (an enzyme in glycolysis) catalyzes the splitting of fructose-1, 6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

Isomerases

They catalyze the formation of an isomer of a compound. Example: phosphoglucomutase catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate (phosphate group is transferred from one to another position in the same compound) in glycogenolysis (glycogen is converted to glucose for energy to be released quickly).

Ligases

Ligases catalyze the association of two molecules. For example, DNA ligase catalyzes the joining of two fragments of DNA by forming a phosphodiester bond.

Cofactors

Cofactors are non-proteinous substances that associate with enzymes. A cofactor is essential for the functioning of an enzyme. The protein part of enzymes in cofactors is apoenzyme. An enzyme and its cofactor together constitute the holoenzyme.

There are three kinds of cofactors present in enzymes:

- **Prosthetic groups**: These are cofactors tightly bound to an enzyme at all times. FAD (flavin adenine dinucleotide) is a prosthetic group present in many enzymes.
- **Coenzyme**: A coenzyme binds to an enzyme only during catalysis. At all other times, it is detached from the enzyme. NAD is a common coenzyme.
- **Metal ions**: For the catalysis of certain enzymes, a metal ion is required at the active site to form coordinate bonds. Zinc is a metal ion cofactor used by a number of enzymes.

Control of Metabolism through Enzyme Regulation

Cellular needs and conditions vary from cell to cell and change within individual cells over time. For example, a stomach cell requires a different amount of energy than a skin cell, fat storage cell, blood cell, or nerve cell. The same stomach cell may also need more energy immediately after a meal and less energy between meals.

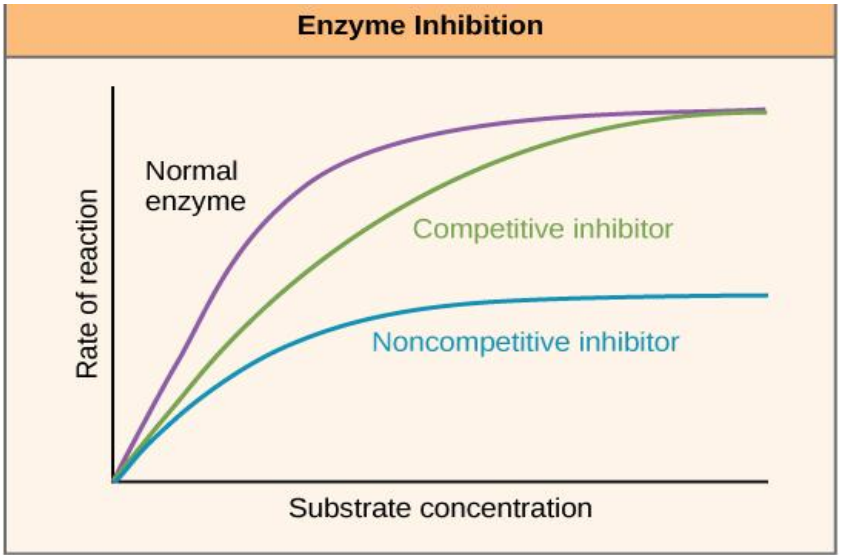


Figure: Enzyme inhibition**:** Competitive and noncompetitive inhibition affects the rate of reaction differently. Competitive inhibitors affect the initial rate, but do not affect the maximal rate, whereas noncompetitive inhibitors affect the maximal rate.

A cell's function is encapsulated by the chemical reactions it can carry out. Enzymes lower the activation energies of chemical reactions; in cells, they promote those reactions that are specific to the cell's function. Because enzymes ultimately determine which chemical reactions a cell can carry out and the rate at which they can proceed, they are key to cell functionality.

Competitive and Noncompetitive Inhibition

The cell uses specific molecules to regulate enzymes in order to promote or inhibit certain chemical reactions. Sometimes it is necessary to inhibit an enzyme to reduce a reaction rate, and there is more than one way for this inhibition to occur. In competitive inhibition, an inhibitor molecule is similar enough to a substrate that it can bind to the enzyme's active site

to stop it from binding to the substrate. It “competes” with the substrate to bind to the enzyme.

In noncompetitive inhibition, an inhibitor molecule binds to the enzyme at a location other than the active site (an allosteric site). The substrate can still bind to the enzyme, but the inhibitor changes the shape of the enzyme so it is no longer in optimal position to catalyze the reaction.

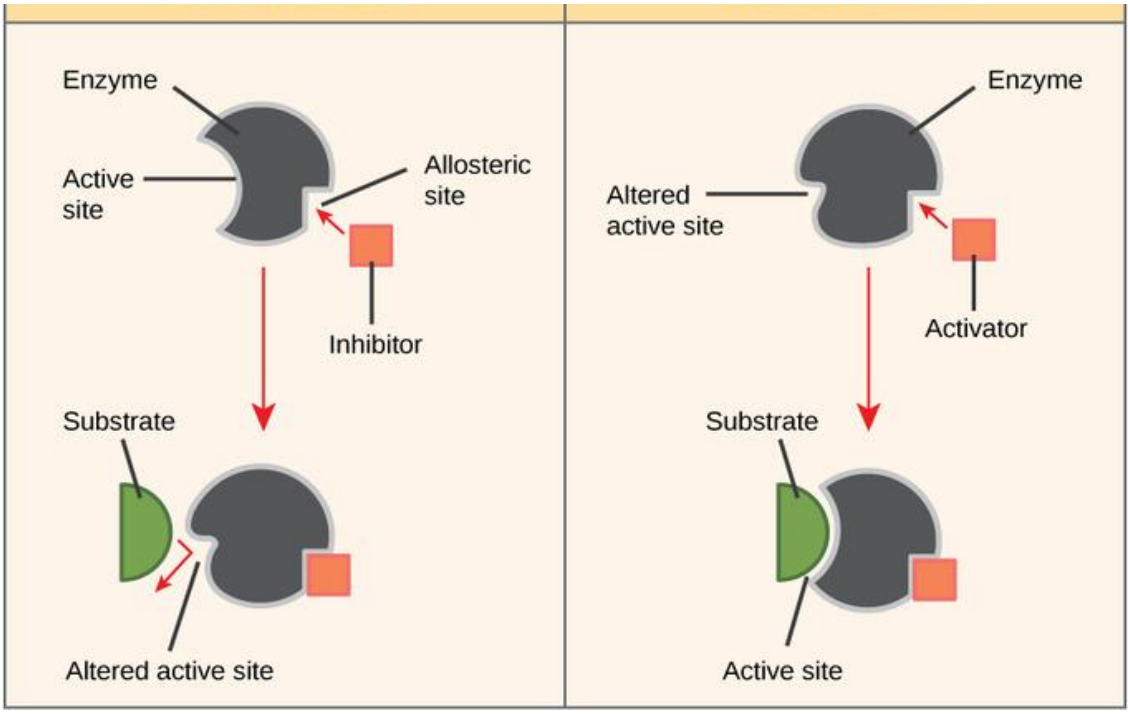
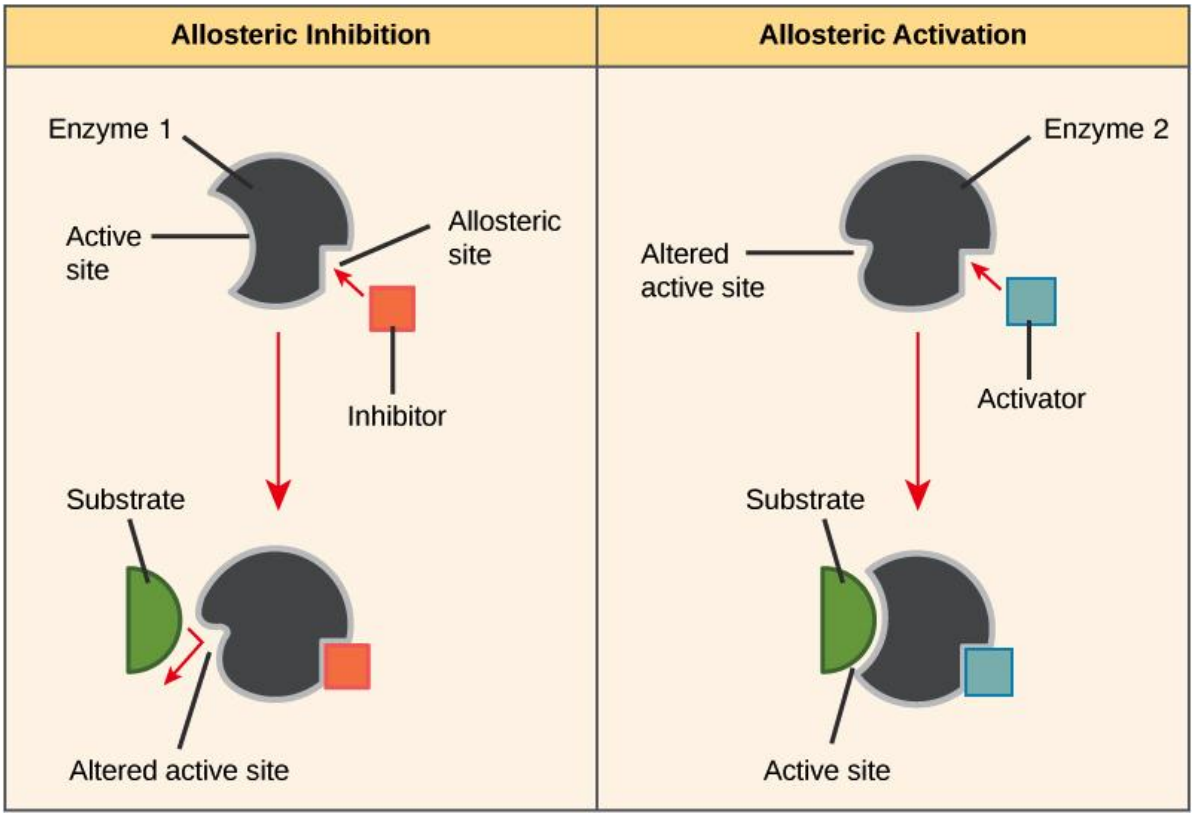


Figure: **Allosteric inhibitors and activators**: Allosteric inhibitors modify the active site of the enzyme so that substrate binding is reduced or prevented. In contrast, allosteric activators modify the active site of the enzyme so that the affinity for the substrate increases.

Allosteric Inhibition and Activation

In noncompetitive allosteric inhibition, inhibitor molecules bind to an enzyme at the allosteric site. Their binding induces a conformational change that reduces the affinity of the enzyme's active site for its substrate. The binding of this allosteric inhibitor changes the conformation of the enzyme and its active site, so the substrate is not able to bind. This prevents the enzyme from lowering the activation energy of the reaction, and the reaction rate is reduced.

However, allosteric inhibitors are not the only molecules that bind to allosteric sites. Allosteric activators can increase reaction rates. They bind to an allosteric site which induces a conformational change that *increases* the affinity of the enzyme's active site for its substrate. This increases the reaction rate.

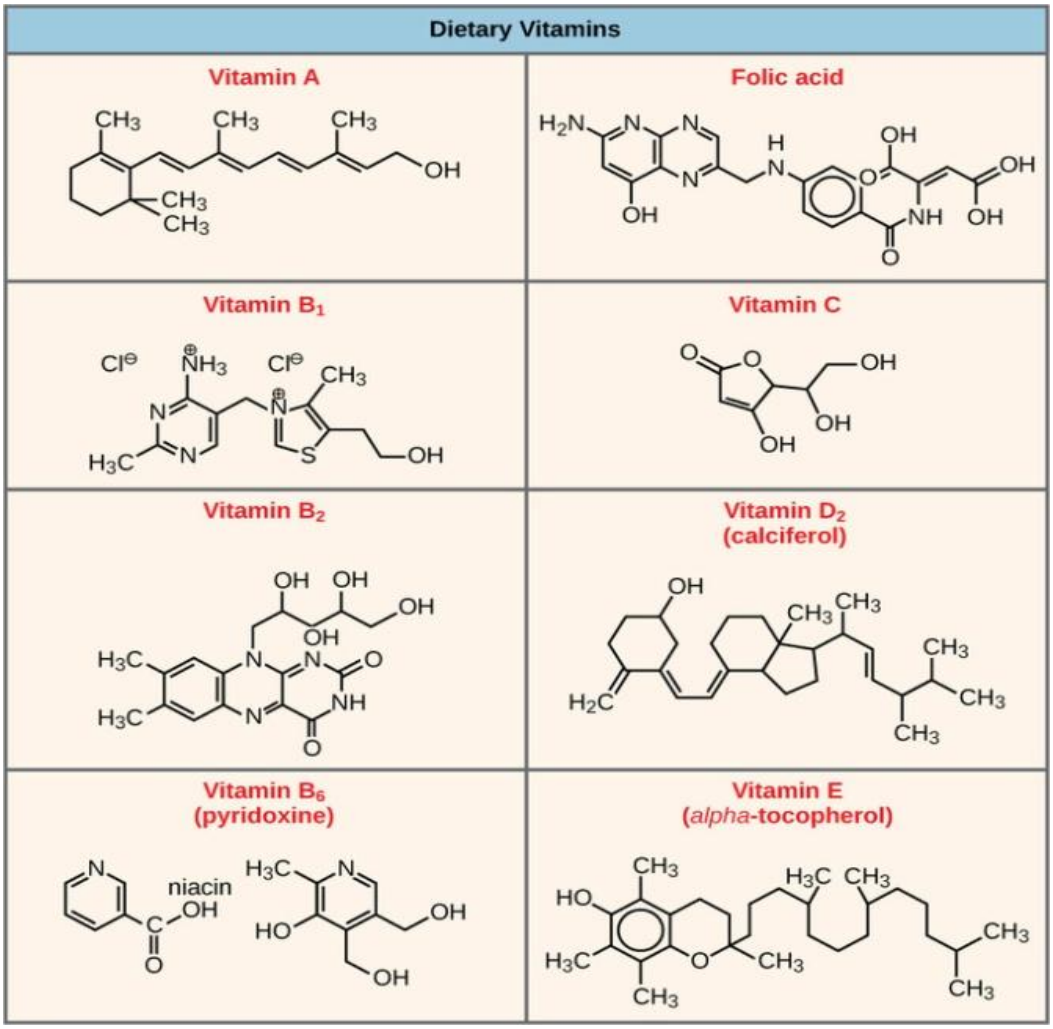


Enzyme regulation

Cofactors and Coenzymes

Many enzymes only work if bound to non-protein helper molecules called cofactors and coenzymes. Binding to these molecules promotes optimal conformation and function for their respective enzymes. These molecules bind temporarily through ionic or hydrogen bonds or permanently through stronger covalent bonds.

Cofactors are inorganic ions such as iron (Fe^{2+}) and magnesium (Mg^{2+}). For example, DNA polymerase requires a zinc ion (Zn^{2+}) to build DNA molecules. Coenzymes are organic helper molecules with a basic atomic structure made up of carbon and hydrogen. The most common coenzymes are dietary vitamins. Vitamin C is a coenzyme for multiple enzymes that take part in building collagen, an important component of connective tissue.



Pyruvate dehydrogenase is a complex of several enzymes that requires one cofactor and five different organic coenzymes to catalyze its chemical reaction. The availability of various cofactors and coenzymes regulates enzyme function.

Figure: **Vitamins**: Vitamins are important coenzymes or precursors of coenzymes and are required for enzymes to function properly. Multivitamin capsules usually contain mixtures of all the vitamins at different percentages.

Enzyme Compartmentalization

In eukaryotic cells, molecules such as enzymes are usually compartmentalized into different organelles. This organization contributes to enzyme regulation because certain cellular processes are contained in separate organelles. For example, the enzymes involved in the later stages of cellular respiration carry out reactions exclusively in the mitochondria. The enzymes involved in the digestion of cellular debris and foreign materials are located within lysosomes.

Feedback Inhibition in Metabolic Pathways

Feedback inhibition is when a reaction product is used to regulate its own further production. Cells have evolved to use feedback inhibition to regulate enzyme activity in metabolism, by using the products of the enzymatic reactions to inhibit further enzyme activity. Metabolic reactions, such as anabolic and catabolic processes, must proceed according to the demands of the cell. In order to maintain chemical equilibrium and meet the needs of the cell, some metabolic products inhibit the enzymes in the chemical pathway while some reactants activate them.

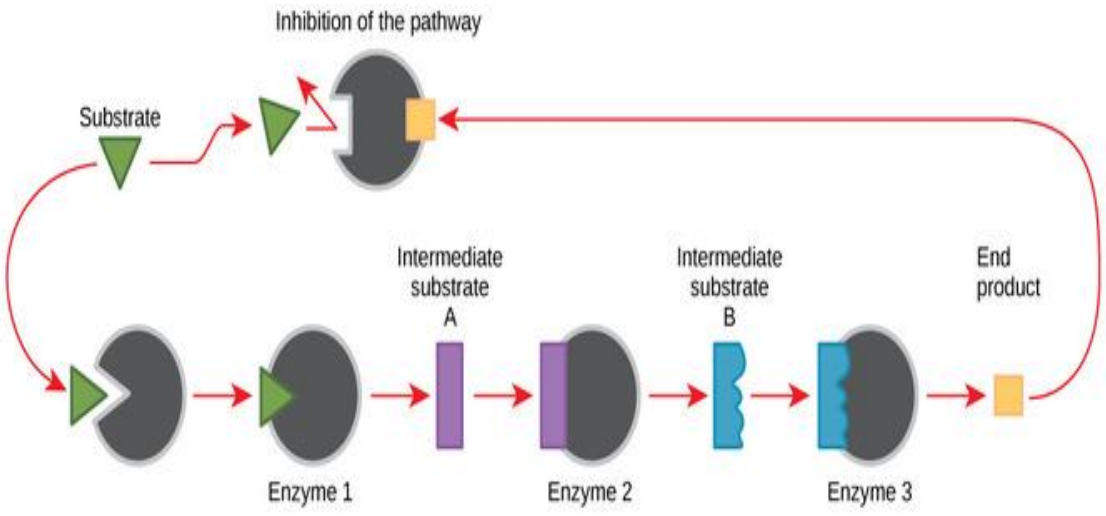


Figure: Feedback inhibition**:** Metabolic pathways are a series of reactions catalyzed by multiple enzymes. Feedback inhibition, where the end product of the pathway inhibits an earlier step, is an important regulatory mechanism in cells.

The production of both amino acids and nucleotides is controlled through feedback inhibition. For an example of feedback inhibition, consider ATP. It is the product of the catabolic metabolism of sugar (cellular respiration), but it also acts as an allosteric regulator for the same enzymes that produced it. ATP is an unstable molecule that can spontaneously dissociate into ADP; if too much ATP were present, most of it would go to waste. This feedback inhibition prevents the production of additional ATP if it is already abundant. However, while ATP is an inhibitor, ADP is an allosteric activator. When levels of ADP are high compared to ATP levels, ADP triggers the catabolism of sugar to produce more ATP.

- The enzyme ‘s active site binds to the substrate.
- Increasing the temperature generally increases the rate of a reaction, but dramatic changes in temperature and pH can denature an enzyme, thereby abolishing its action as a catalyst.
- The induced fit model states an substrate binds to an active site and both change shape slightly, creating an ideal fit for catalysis.
- When an enzyme binds its substrate it forms an enzyme-substrate complex.
- Enzymes promote chemical reactions by bringing substrates together in an optimal orientation, thus creating an ideal chemical environment for the reaction to occur.
- The enzyme will always return to its original state at the completion of the reaction.

Enzyme Active Site and Substrate Specificity

Enzymes bind with chemical reactants called substrates. There may be one or more substrates for each type of enzyme, depending on the particular chemical reaction. In some reactions, a single-reactant substrate is broken down into multiple products. In others, two substrates may come together to create one larger molecule. Two reactants might also enter a reaction both become modified, and leave the reaction as two products.

The enzyme's active site binds to the substrate. Since enzymes are proteins, this site is composed of a unique combination of amino acid residues (side chains or R groups). Each amino acid residue can be large or small; weakly acidic or basic; hydrophilic or hydrophobic; and positively-charged, negatively-charged, or neutral. The positions, sequences, structures, and properties of these residues create a very specific chemical environment within the active site. A specific chemical substrate matches this site like a jigsaw puzzle piece and makes the enzyme specific to its substrate.

Active Sites and Environmental Conditions

Environmental conditions can affect an enzyme's active site and, therefore, the rate at which a chemical reaction can proceed. Increasing the environmental temperature generally increases reaction rates because the molecules are moving more quickly and are more likely to come into contact with each other.However, increasing or decreasing the temperature outside of an optimal range can affect chemical bonds within the enzyme and change its shape. If the enzyme changes shape, the active site may no longer bind to the appropriate substrate and the rate of reaction will decrease. Dramatic changes to the temperature and pH will eventually cause enzymes to denature.

Induced Fit and Enzyme Function

For many years, scientists thought that enzyme-substrate binding took place in a simple "lock-and-key" fashion. This model asserted that the enzyme and substrate fit together perfectly in one instantaneous step. However, current research supports a more refined view called induced fit. As the enzyme and substrate come together, their interaction causes a mild shift in the enzyme's structure that confirms an ideal binding arrangement between the enzyme and the substrate. This dynamic binding maximizes the enzyme's ability to catalyze its reaction.

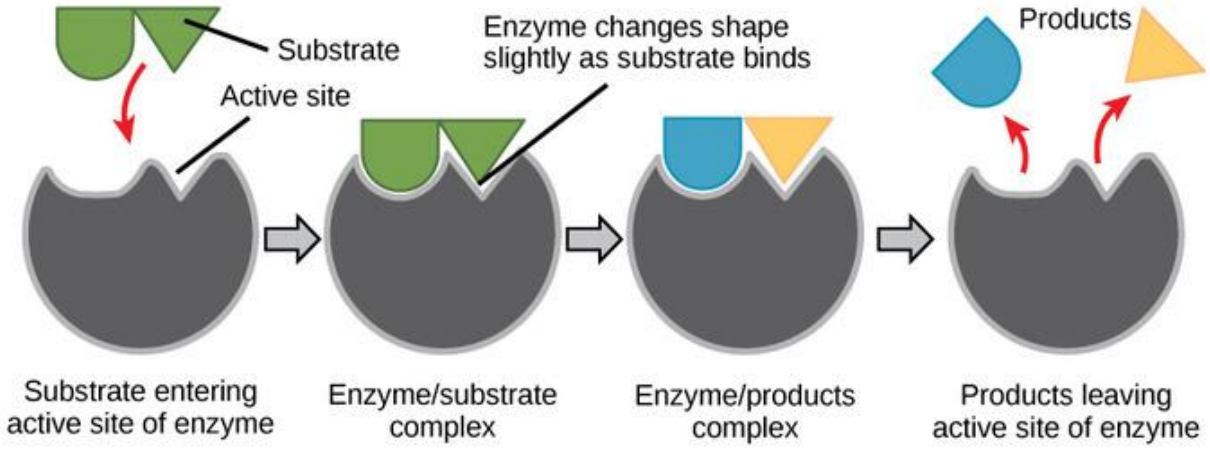


Figure: Induced Fit: According to the induced fit model, both enzyme and substrate undergo dynamic conformational changes upon binding. The enzyme contorts the substrate into its transition state, thereby increasing the rate of the reaction.

Enzyme-Substrate Complex

When an enzyme binds its substrate, it forms an enzyme-substrate complex. This complex lowers the activation energy of the reaction and promotes its rapid progression by providing certain ions or chemical groups that actually form covalent bonds with molecules as a necessary step of the reaction process. Enzymes also promote chemical reactions by bringing substrates together in an optimal orientation, lining up the atoms and bonds of one molecule with the atoms and bonds of the other molecule. This can contort the substrate molecules and facilitate bond-breaking. The active site of an enzyme also creates an ideal environment, such as a slightly acidic or non-polar environment, for the reaction to occur. The enzyme will always return to its original state at the completion of the reaction. One of the important properties of enzymes is that they remain ultimately unchanged by the reactions they catalyze. After an enzyme is done catalyzing a reaction, it releases its products (substrates).

Extraction, Assay, Purification:

Isolating different enzymes from cells or crude extracts which contain more components other than enzymes has been known as enzyme purification. This process of purification has been seen to be done in order to get maximum activity from initial activity based on appropriate recovery possibilities. Several processes have been seen to be present which are used widely in enzyme purification however, selecting a proper process in the treatment

stage should always be of top priority.

Isolation of enzymes has been known as a process of differentiating enzymes from crude sources or cells. Isolation and purification of enzymes can be helpful in differentiating enzymes and calculating their activity and recovery percentage. Isolation of enzymes can be done in some simple steps which can be cell disruption, supernatant removal or centrifugation. Cell disruption can be done using osmolysis, freeze-thaw cycles, ultrasonication, detergent lysis, enzymatic lysis or homogenisation. Supernatant removal can be done using a decantation process which has been a widely known process of obtaining supernatant. Centrifugation has been known as a process that needs to be conducted at a specific speed based on tissue, cells or other materials from where enzymes need to be isolated.

Purification of enzymes after being isolated can be done using five simple steps. These steps include Dialysis, Ion exchange chromatography, Salt precipitation using (NH4)SO4, Size-exclusion chromatography and Affinity chromatography. Proper Isolation and purification of enzymes can provide activity measurement of an enzyme.

Total enzyme activity = Change in substrate concentration / Time taken

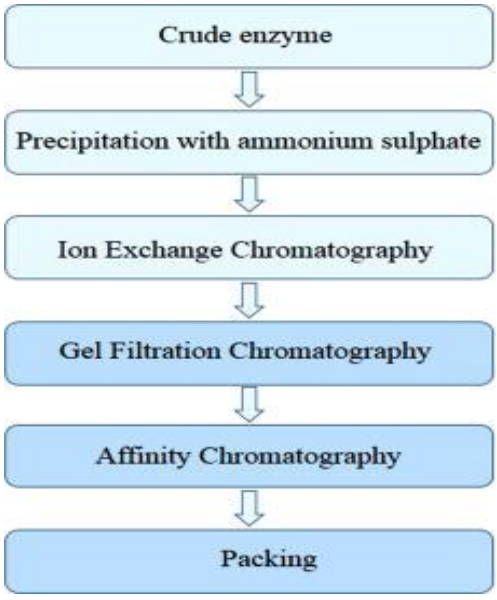
This has also been helpful in determining recovery rate.

Recovery rate = (Total enzyme activity in purified fraction / Total enzyme activity in crude homogenate) X 100%

Enzymes are mainly a type of protein and a protein needs to be isolated in a pure state for analysis. Isolation and purification of enzymes can be done using some simple steps and these steps include precipitation, concentration, extraction, purification and storage. Isolation of enzymes can be done using ultrasound homogenisation, cryogenic grinding and lysis buffer. Ultrasound homogenisation can be used for isolating enzymes from soft tissues and this process has also been known to cause heat of the sample. Cryogenic grinding includes grinding of samples in liquid nitrogen. This process, which includes liquid nitrogen, has been seen to have a very lower temperature which protects enzymes during grinding. This process has mainly been seen to be used for isolating enzymes from hard tissues however; this process has also been seen to be time-consuming. Lysis buffer can be used for isolating enzymes from only animal cells or bacteria. Risk of degradation has been seen to be present in this process.

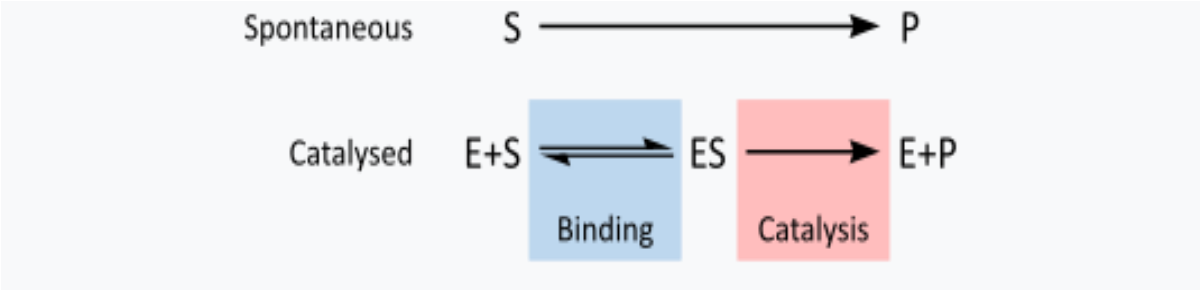
Purification of enzymes has also been known as separation of enzymes from other substances. Purification of enzymes can be done in three types and those are purification by charge, purification by size and purification by specific binding sites. Purification by charge includes isoelectric focusing and exchange chromatography whereas purification by size includes size exclusion chromatography, preparative native gel electrophoresis and ultrafiltration. Purification by binding sites includes immunochromatography using antibodies, magnetic separation using magnetic antibodies and metal affinity chromatography using different binding sites.

Isolation and purification of enzymes have been known as processes that can be used to isolate enzymes from a mixture of enzymes. Purification of enzymes can be useful in identifying structure, function and interactions of a specific enzyme. Before starting purification process extraction, centrifugation and precipitation are needed in order to help purification process generate best results. Purification processes of enzymes include size exclusion chromatography, ion-exchange chromatography, hydrophobic interaction chromatography, free-flow-electrophoresis, affinity chromatography and High-performance liquid chromatography (HPLC). Purification of an enzyme completes when Ultrafiltration and Lyophilization have been used and concentration of purified enzyme has been gained. Isolation and Purification of enzymes can also provide proper evaluation of purification yield.

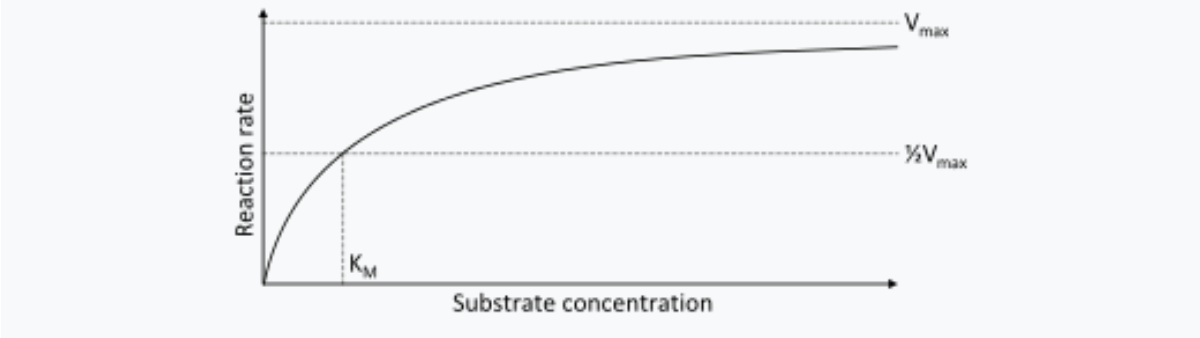


Enzyme Purification

Michaelis–Menten kinetics



A chemical reaction mechanism with or without enzyme catalysis. The enzyme (E) binds substrate (S) to produce product (P).



Saturation curve for an enzyme reaction showing the relation between the substrate concentration and reaction rate.

As enzyme-catalysed reactions are saturable, their rate of catalysis does not show a linear response to increasing substrate. If the initial rate of the reaction is measured over a range of substrate concentrations the initial reaction rate (increases as increases. However, as gets higher, the enzyme becomes saturated with substrate and the initial rate reaches V_{max}, the enzyme's maximum rate.

The Michaelis–Menten kinetic model of a single-substrate reaction is shown on the right. There is an initial bimolecular reaction between the enzyme E and substrate S to form the enzyme–substrate complex ES. The rate of enzymatic reaction increases with the increase of the substrate concentration up to a certain level called V_{max}; at V_{max}, increase in substrate concentration does not cause any increase in reaction rate as there is no more enzyme (E) available for reacting with substrate (S). Here, the rate of reaction becomes dependent on the ES complex and the reaction becomes a unimolecular reaction with an order of zero.

Though the enzymatic mechanism for the unimolecular reaction can be quite complex, there is typically one rate-determining enzymatic step that allows this reaction to be modelled as a single catalytic step with an apparent unimolecular rate constant k_{cat}. If the reaction path proceeds over one or several intermediates, k_{cat} will be a function of several elementary rate constants, whereas in the simplest case of a single elementary reaction (e.g. no intermediates) it will be identical to the elementary unimolecular rate constant k_2. The apparent unimolecular rate constant k_{cat} is also called turnover number, and denotes the maximum number of enzymatic reactions catalysed per second.

The Michaelis–Menten equation describes how the (initial) reaction rate v_0 depends on the position of the substrate-binding equilibrium and the rate constant.

This Michaelis–Menten equation is the basis for most single-substrate enzyme kinetics. Two crucial assumptions underlie this equation (apart from the general assumption about the mechanism only involving no intermediate or product inhibition, and there is no allostericity or cooperativity). The first assumption is the so-called quasi-steady-state assumption (or pseudo-steady-state hypothesis), namely that the concentration of the substrate-bound enzyme (and hence also the unbound enzyme) changes much more slowly than those of the product and substrate and thus the change over time of the complex can be set to zero . The second assumption is that the total enzyme concentration does not change over time, thus . A complete derivation can be found here.

The Michaelis constant K_M is experimentally defined as the concentration at which the rate of the enzyme reaction is half V_{max}, which can be verified by substituting $= K_M$ into the Michaelis–Menten equation and can also be seen graphically. If the rate-determining enzymatic step is slow compared to substrate dissociation, the Michaelis constant K_M is roughly the dissociation constant K_D of the ES complex. If is small compared to then the term and also very little ES complex is formed, thus. Therefore, the rate of product formation is

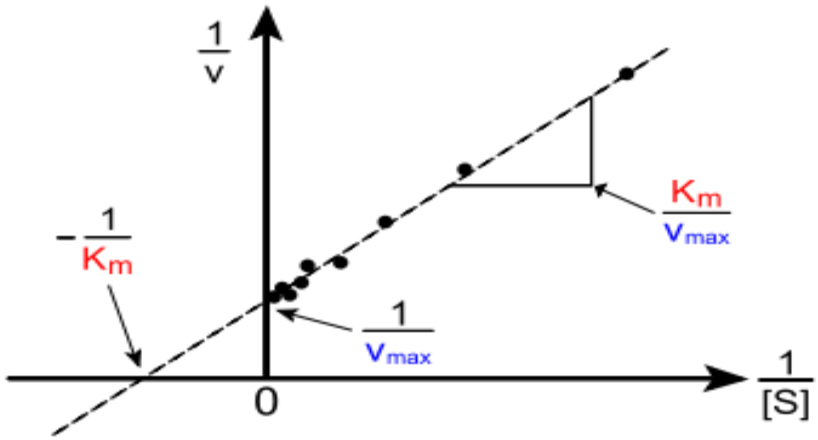
Thus the product formation rate depends on the enzyme concentration as well as on the substrate concentration, the equation resembles a bimolecular reaction with a corresponding pseudo-second order rate constant This constant is a measure of catalytic efficiency. The most efficient enzymes reach in the range of $10^8 - 10^{10}\ M^{-1}\ s^{-1}$. These enzymes are so efficient they effectively catalyse a reaction each time they encounter a substrate molecule and have thus reached an upper theoretical limit for efficiency (diffusion limit).

Direct use of the Michaelis–Menten equation for time course kinetic analysis

The observed velocities predicted by the Michaelis–Menten equation can be used to directly model the time course disappearance of substrate and the production of product through incorporation of the Michaelis–Menten equation into the equation for first order chemical kinetics. This can only be achieved however if one recognises the problem associated with the use of Euler's number in the description of first order chemical kinetics. i.e. e^{-k} is a split constant that introduces a systematic error into calculations and can be rewritten as a single constant which represents the remaining substrate after each time period.

Linear plots of the Michaelis–Menten equation

See also: Lineweaver–Burk plot, Eadie–Hofstee diagram, and Hanes–Woolf plot



Lineweaver–Burk or double-reciprocal plot of kinetic data, showing the significance of the axis intercepts and gradient. The plot of *v* versus above is not linear; although initially linear at low , it bends over to saturate at high . Before the modern era of nonlinear curve-fitting on computers, this nonlinearity could make it difficult to estimate K_M and V_{max} accurately. Therefore, several researchers developed linearisations of the Michaelis–Menten equation, such as the Lineweaver–Burk plot, the Eadie–Hofstee diagram and the Hanes–Woolf plot. All of these linear representations can be useful for visualising data, but none should be used to determine kinetic parameters, as computer software is readily available that allows for more accurate determination by nonlinear regression methods.

The Lineweaver–Burk plot or double reciprocal plot is a common way of illustrating kinetic data. This is produced by taking the reciprocal of both sides of the Michaelis–Menten equation. As shown on the right, this is a linear form of the Michaelis–Menten equation and

produces a straight line with the equation $y = mx + c$ with a y-intercept equivalent to $1/V_{max}$ and an x-intercept of the graph representing $-1/K_M$.

Naturally, no experimental values can be taken at negative 1/; the lower limiting value 1/ = 0 (the y-intercept) corresponds to an infinite substrate concentration, where $1/v=1/V_{max}$ as shown at the right; thus, the x-intercept is an extrapolation of the experimental data taken at positive concentrations. More generally, the Lineweaver–Burk plot skews the importance of measurements taken at low substrate concentrations and, thus, can yield inaccurate estimates of V_{max} and K_M. A more accurate linear plotting method is the Eadie–Hofstee plot. In this case, v is plotted against $v/$. In the third common linear representation, the Hanes–Woolf plot, $/v$ is plotted against. In general, data normalisation can help diminish the amount of experimental work and can increase the reliability of the output, and is suitable for both graphical and numerical analysis.

Practical significance of kinetic constants

The study of enzyme kinetics is important for two basic reasons. Firstly, it helps explain how enzymes work, and secondly, it helps predict how enzymes behave in living organisms. The kinetic constants defined above, K_M and V_{max}, are critical to attempts to understand how enzymes work together to control metabolism.

Making these predictions is not trivial, even for simple systems. For example, oxaloacetate is formed by malate dehydrogenase within the mitochondrion. Oxaloacetate can then be consumed by citrate synthase, phosphoenolpyruvate carboxykinase or aspartate aminotransferase, feeding into the citric acid cycle, gluconeogenesis or aspartic acid biosynthesis, respectively. Being able to predict how much oxaloacetate goes into which pathway requires knowledge of the concentration of oxaloacetate as well as the concentration and kinetics of each of these enzymes. This aim of predicting the behaviour of metabolic pathways reaches its most complex expression in the synthesis of huge amounts of kinetic and gene expression data into mathematical models of entire organisms. Alternatively, one useful simplification of the metabolic modelling problem is to ignore the underlying enzyme kinetics and only rely on information about the reaction network's stoichiometry, a technique called flux balance analysis.

Carbohydrate metabolism: Embden- Meyerhoff-Parnas pathway

EMP pathway is the other name of glycolysis. It is named after the three scientists Gustav Embden, Otto Meyerhof, and J. Parnas, who gave the scheme of glycolysis. It is the pathway of glucose catabolism. It occurs in the cytoplasm of all living cells, aerobic as well as anaerobic. EMP pathway or glycolysis is the primary step of cellular respiration. Glucose is partially oxidised to pyruvate in this process. In aerobic organisms, it is followed by the Krebs cycle for the complete oxidation of glucose to CO_2 and water. In anaerobic organisms, glycolysis is followed by fermentation. Let us learn in detail about each step of the EMP pathway.

Steps of EMP Pathway

The EMP pathway occurs in the cytoplasm of the cell. It doesn't require oxygen. In plants, glucose is derived from sucrose formed during photosynthesis or from storage carbohydrates such as starch. The enzyme invertase converts sucrose into glucose and fructose that enter the EMP pathway.

- It is a series of ten enzyme-catalysed reactions, wherein a glucose molecule is broken down into two molecules of pyruvate.
- There is a net production of two ATP and 2 NADH also in this process.
- The first phase of the EMP pathway is the energy-requiring phase or preparatory phase, and the second half is the energy-yielding phase or pay-off phase.

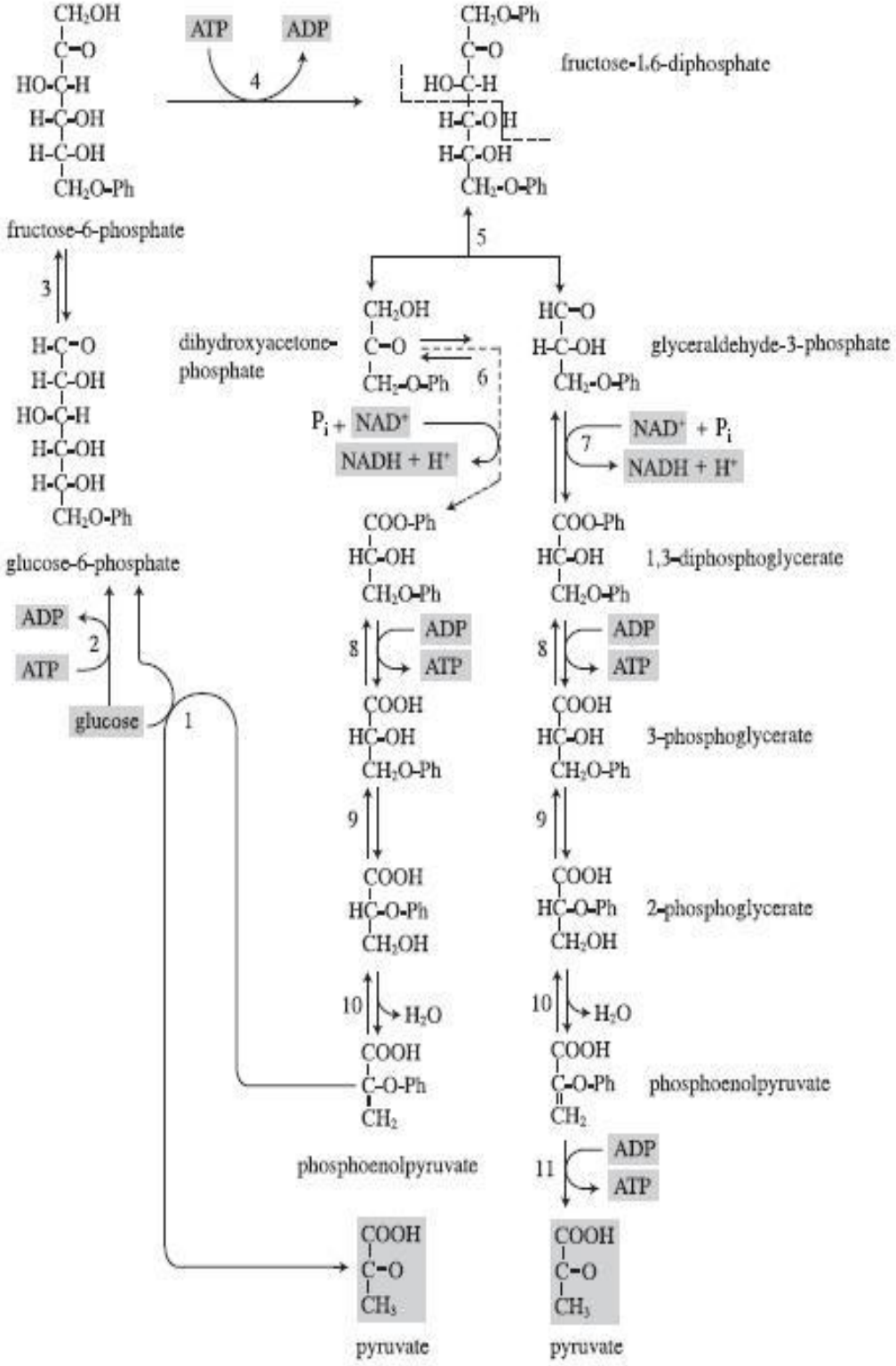


Fig: EMP pathwa

TCA Cycle:

Like the conversion of pyruvate to acetyl CoA, the citric acid cycle takes place in the matrix of the mitochondria. Almost all of the enzymes of the citric acid cycle are soluble, with the single exception of the enzyme succinate dehydrogenase, which is embedded in the inner membrane of the mitochondrion. Unlike glycolysis, the citric acid cycle is a closed loop: the last part of the pathway regenerates the compound used in the first step. The eight steps of the cycle are a series of redox, dehydration, hydration, and decarboxylation reactions that produce two carbon dioxide molecules, one GTP/ATP, and reduced forms of NADH and FADH2. This is considered an aerobic pathway because the NADH and FADH2 produced must transfer their electrons to the next pathway in the system, which will use oxygen. If this transfer does not occur, the oxidation steps of the citric acid cyclealso do not occur. Note that the citric acid cycle produces very little ATP directly and does not directly consume oxygen.

Steps in the Citric Acid Cycle

Step 1. The first step is a condensation step, combining the two-carbon acetyl group (from acetyl CoA) with a four-carbon oxaloacetate molecule to form a six-carbon molecule of citrate. CoA is bound to a sulfhydryl group (-SH) and diffuses away to eventually combine with another acetyl group. This step is irreversible because it is highly exergonic. The rate of this reaction is controlled by negative feedback and the amount of ATP available. If ATP levels increase, the rate of this reaction decreases. If ATP is in short supply, the rate increases.

Step 2. Citrate loses one water molecule and gains another as citrate is converted into its isomer, isocitrate.

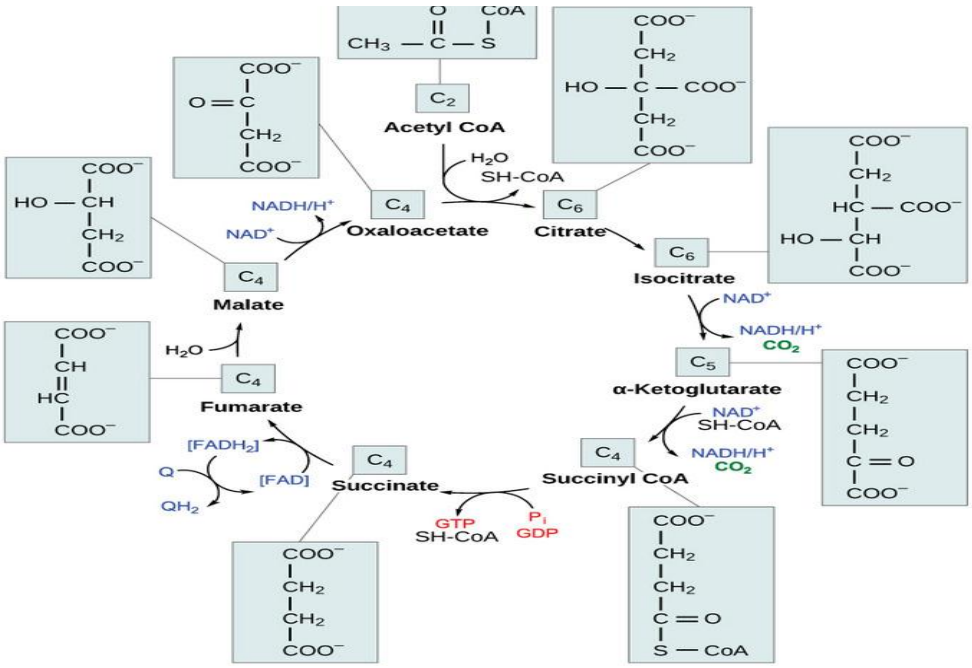
Steps 3 and 4. In step three, isocitrate is oxidized, producing a five-carbon molecule, α-ketoglutarate, together with a molecule of CO_2 and two electrons, which reduce NAD+ to NADH. This step is also regulated by negative feedback from ATP and NADH and by a positive effect of ADP. Steps three and four are both oxidation and decarboxylation steps, which release electrons that reduce NAD^+ to NADH and release carboxyl groups that form CO_2 molecules. α-Ketoglutarate is the product of step three, and a succinyl group is the product of step four. CoA binds the succinyl group to form succinyl CoA. The enzyme that catalyzes step four is regulated by feedback inhibition of ATP, succinyl CoA, and NADH.

Step 5. A phosphate group is substituted for coenzyme A, and a high- energy bond is formed. This energy is used in substrate-level phosphorylation (during the conversion of the succinyl group to succinate) to form either guanine triphosphate (GTP) or ATP. There are two forms of the enzyme, called isoenzymes, for this step, depending upon the type of animal tissue in which they are found. One form is found in tissues that use large amounts of ATP, such as heart and

skeletal muscle. This form produces ATP. The second form of the enzyme is found in tissues that have a high number of anabolic pathways, such as liver. This form produces GTP. GTP is energetically equivalent to ATP; however, its use is more restricted. In particular, protein synthesis primarily uses GTP.

Step 6. Step six is a dehydration process that converts succinate into fumarate. Two hydrogen atoms are transferred to FAD, producing $FADH_2$. The energy contained in the electrons of these atoms is insufficient to reduce NAD^+ but adequate to reduce FAD. Unlike NADH, this carrier remains attached to the enzyme and transfers the electrons to the electron transport chain directly. This process is made possible by the localization of the enzyme catalyzing this step inside the inner membrane of the mitochondrion.

Step 7. Water is added to fumarate during step seven, and malate is produced. The last step in the citric acid cycle regenerates oxaloacetate by oxidizing malate. Another molecule of NADH is produced.



Products of the Citric Acid Cycle

Two carbon atoms come into the citric acid cycle from each acetyl group, representing four out of the six carbons of one glucose molecule. Two carbon dioxide molecules are released on each turn of the cycle; however, these do not necessarily contain the most recently-added carbon atoms.

The two acetyl carbon atoms will eventually be released on later turns of the cycle; thus, all six carbon atoms from the original glucose molecule are eventually incorporated into carbon dioxide. Each turn of the cycle forms three NADH molecules and one $FADH_2$ molecule. These carriers will connect with the last portion of aerobic respiration to produce ATP molecules. One GTP or ATP is also made in each cycle. Several of the intermediate compounds in the citric acid cycle can be used in synthesizing non-essential amino acids; therefore, the cycle is amphibolic (both catabolic and anabolic).

Pentose Phosphate Pathway:

The pentose phosphate pathway (PPP; also called the phosphogluconate pathway and the hexose monophosphate shunt) is a process that breaks down glucose-6-phosphate into NADPH and pentoses (5-carbon sugars) for use in downstream biological processes. There are two distinct phases in the pathway: the oxidative phase and the non-oxidative phase. The first is the oxidative phase in which glucose-6-phosphate is converted to ribulose-5-phosphate. During this process two molecules of $NADP^+$are reduced to NADPH. The overall reaction for this process is:

Figure 1 The Pentose Phosphate Pathway*:* The pentose phosphate pathway generates reducing equivalents in the form of NADPH. It is used in reductive biosynthesis reactions within cells (e.g. fatty acid synthesis). It produces ribulose-5-phosphate, used in the synthesis of nucleotides. It also produces nucleic acids and erythrose-4-phosphate, used in the synthesis of aromatic amino acids.

Glucose 6-phosphate + 2 $NADP^+$ + H_2O → ribulose-5-phosphate + 2 NADPH + 2 H^+ + CO_2

The second phase of this pathway is the non-oxidative synthesis of 5-carbon sugars. Depending on the body's state, ribulose-5-phosphate can reversibly isomerize to ribose-5-phosphate. Ribulose-5-phosphate can alternatively undergo a series of isomerizations as well as transaldolations and transketolations that result in the production of other pentose phosphates including fructose-6-phosphate, erythrose-4-phosphate, and glyceraldehyde-3-phosphate (both intermediates in glycolysis). These compounds are used in a variety of different biological processes including production of nucleotides and nucleic acids (ribose-5-phosphate), as well as synthesis of aromatic amino acids (erythrose-4-phosphate).

Glucose-6-phosphate dehydrogenase is the rate-controlling enzyme in this pathway. It is allosterically stimulated by $NADP^+$. NADPH-utilizing pathways, such as fatty acid synthesis, generate $NADP^+$, which stimulates glucose-6-phosphate dehydrogenase to produce more NADPH. In mammals, the PPP occurs exclusively in the cytoplasm; it is found to be most active in the liver, mammary gland, and adrenal cortex. The ratio of NADPH: $NADP^+$ is normally about 100:1 in liver cytosol, making the cytosol a highly-reducing environment.

The PPP is one of the three main ways the body creates molecules with reducing power, accounting for approximately 60% of NADPH production in humans. While the PPP does involve oxidation of glucose, its primary role is anabolic rather than catabolic, using the energy stored in NADPH to synthesize large, complex molecules from small precursors.

Additionally, NADPH can be used by cells to prevent oxidative stress. NADPH reduces glutathione via glutathione reductase, which converts reactive H_2O_2 into H_2O by glutathione peroxidase. For example, erythrocytes generate a large amount of NADPH through the pentose phosphate pathway to use in the reduction of glutathione.

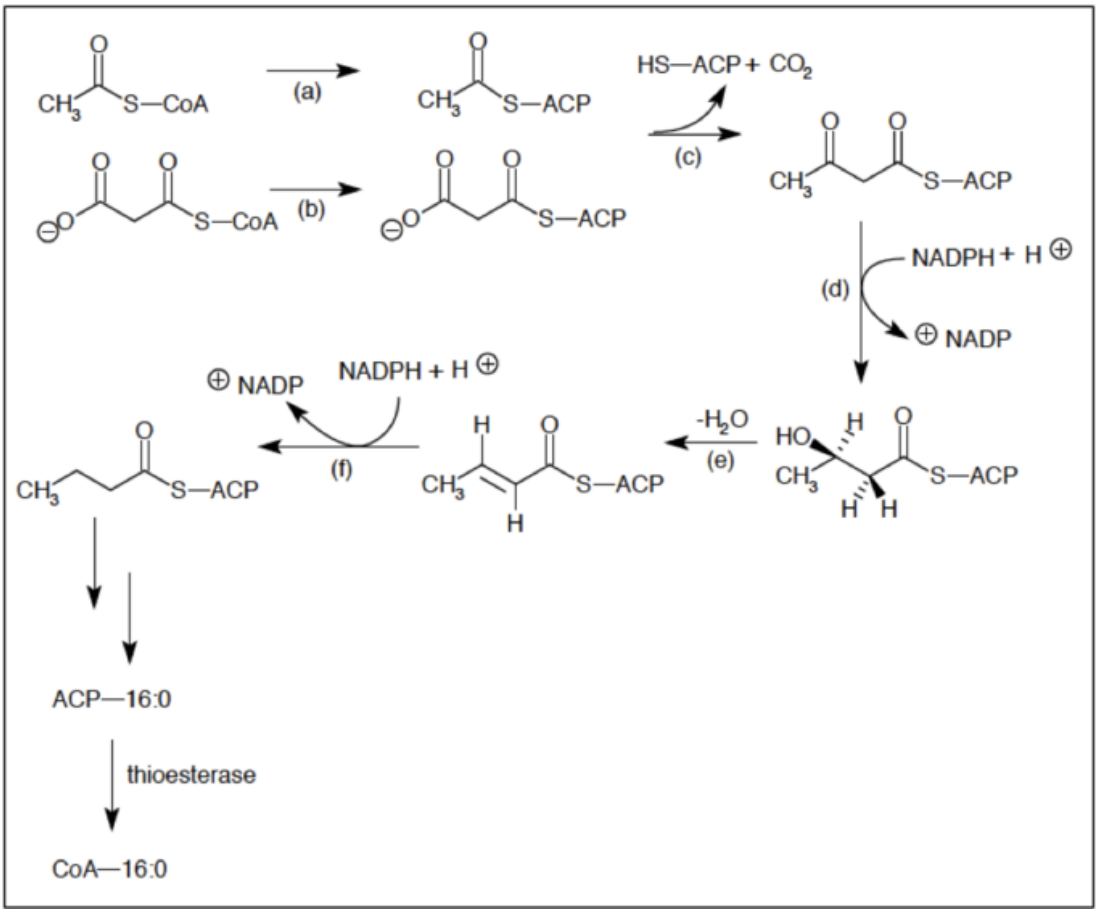


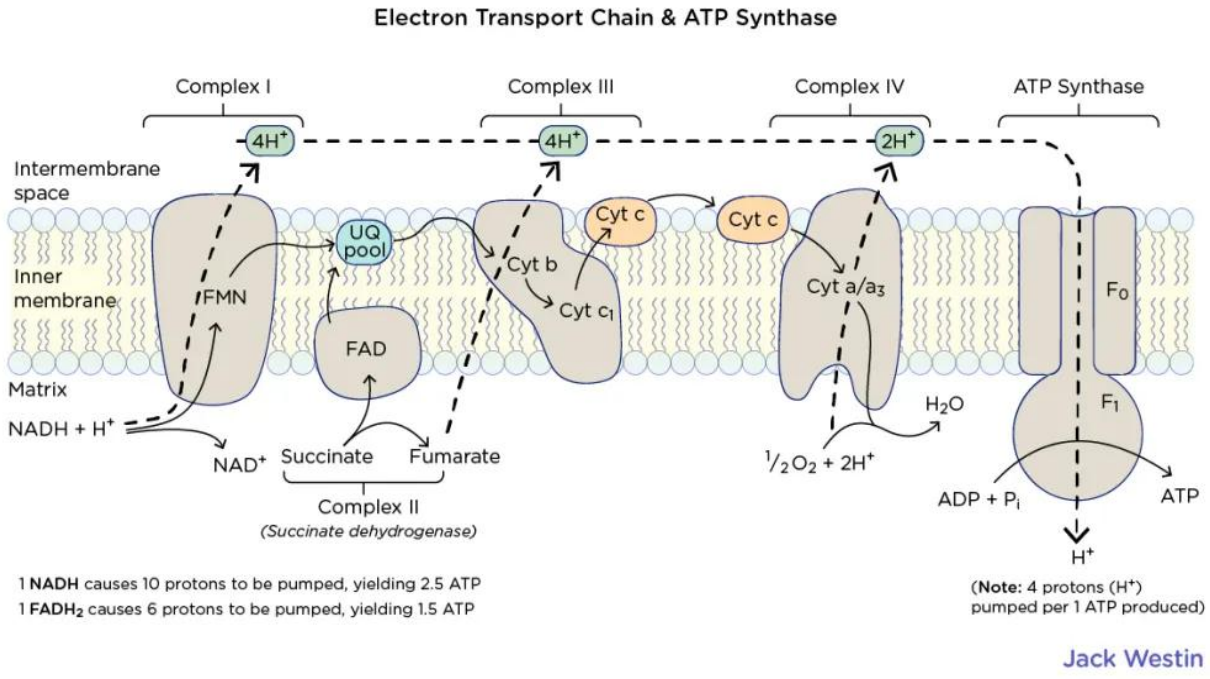
Figure: Fatty Acid Synthesis: Synthesis of Straight-Chain Saturated Fatty Acids

The electron transport chain: is a series of four protein complexes that couple redox reactions, creating an electrochemical gradient that leads to the creation of ATP in a complete system named oxidative phosphorylation. It occurs in mitochondria in both cellular respiration and photosynthesis. In the former, the electrons come from breaking down organic molecules, and energy is released. In the latter, the electrons enter the chain after being excited by light, and the energy released is used to build carbohydrates.

Aerobic cellular respiration is made up of three parts: glycolysis, the citric acid (Krebs) cycle, and oxidative phosphorylation. In glycolysis, glucose metabolizes into two molecules of pyruvate, with an output of ATP and nicotinamide adenine dinucleotide (NADH). Each pyruvate oxidizes into acetyl CoA and an additional molecule of NADH and carbon dioxide (CO2). The acetyl CoA is then used in the citric acid cycle, which is a chain of chemical reactions that produce CO2, NADH, flavin adenine dinucleotide (FADH2), and ATP. In the final step, the three NADH and one FADH2 amassed from the previous steps are used in oxidative phosphorylation, to make water and ATP.

Oxidative phosphorylation has two parts: the electron transport chain (ETC) and chemiosmosis. The ETC is a collection of proteins bound to the inner mitochondrial membrane and organic molecules, which electrons pass through in a series of redox reactions, and release energy. The energy released forms a proton gradient, which is used in chemiosmosis to make a large amount of ATP by the protein ATP-synthase.

Photosynthesis is a metabolic process that converts light energy into chemical energy to build sugars. In the light-dependent reactions, light energy and water are used to make ATP, NADPH, and oxygen (O2). The proton gradient used to make the ATP forms via an electron transport chain. In the light-independent reactions, sugar is made from the ATP and NADPH from the previous reactions.



Electron Transport Chain And Oxidative Phosphorylation Substrates And Products

Cellular Level

In the electron transport chain (ETC), the electrons go through a chain of proteins that increases its reduction potential and causes a release in energy. Most of this energy is dissipated as heat or utilized to pump hydrogen ions (H+) from the mitochondrial matrix to the intermembrane space and create a proton gradient. This gradient increases the acidity in the intermembrane space and creates an electrical difference with a positive charge outside and a negative charge inside. The ETC proteins in a general order are complex I, complex II, coenzyme Q, complex III, cytochrome C, and complex IV.

- **Complex I,** also known as ubiquinone oxidoreductase, is made up of NADH dehydrogenase, flavin mononucleotide (FMN), and eight iron-sulfur (Fe-S) clusters. The NADH donated from glycolysis, and the citric acid cycle is oxidized here, transferring 2 electrons from NADH to FMN. Then they are transferred to the Fe-S clusters and finally from Fe-S to coenzyme Q. During this process, 4 hydrogen ions pass from the mitochondrial matrix to the intermembrane space, contributing to the electrochemical gradient. Complex I may also play an important role in causing apoptosis in programmed cell death.
    - (NADH + H+) + CoQ + 4 H+(matrix) -> NAD+ + CoQH2 + 4 H+(intermembrane)
    - **Complex II,** also known as succinate dehydrogenase, accepts electrons from succinate (an intermediate in the citric acid cycle) and acts as a second entry point to the ETC. When succinate oxidizes to fumarate, 2 electrons are accepted by FAD within complex II. FAD passes them to Fe-S clusters and then to coenzyme Q, similar to complex I. However; no protons are translocated across the membrane by complex II, therefore less ATP is produced with this pathway.
        - Succinate + FAD -> Fumarate + 2 H+(matrix) + FADH2
        - FADH2 + CoQ -> FAD + CoQH2
    - Glycerol-3-Phosphate dehydrogenase and Acyl-CoA dehydrogenase also accept electrons from glycerol-3-P and fatty acyl-CoA, respectively. Inclusion of these protein complexes allows for the donation to the ETC by cytosolic NADH (glycerol-3-P acts as a shuttle to regenerate cytosolic NAD from NADH) and fatty acids undergoing beta-oxidation within the mitochondria (acyl-CoA is oxidized to enoyl-CoA in the first step, producing FADH2).

- Coenzyme Q, also known as ubiquinone (CoQ), is made up of quinone and a hydrophobic tail. Its purpose is to function as an electron carrier and transfer electrons to complex III. Coenzyme Q undergoes reduction to semiquinone (partially reduced, radical form CoQH-) and ubiquinol (fully reduced CoQH2) through the Q cycle. This process receives further elaboration under Complex III.

- **Complex III,** also known as cytochrome c reductase, is made up of cytochrome b, Rieske subunits (containing two Fe-S clusters), and cytochrome c proteins. A cytochrome is a protein involved in electron transfer that contains a heme group. The heme groups alternate between ferrous (Fe2+) and ferric (Fe3+) states during the electron transfer. Because cytochrome c can only accept a single electron at a time, this process occurs in two steps (the Q cycle), in contrast to the single-step complex I and II pathways. Complex III also releases 4 protons into the intermembrane space at the end of a full Q cycle, contributing to the gradient. Cytochrome c then transfers the electrons one at a time to complex IV.

  - **Q Cycle:**

Step 1 in the Q cycle involves ubiquinol (CoQH2) and ubiquinone (CoQ) binding to two separate sites on complex III. CoQH2 transfers each electron to a different path. One electron goes to Fe-S and then cytochrome c, while the second electron is transferred to cytochrome b and then to CoQ bound at the other site. While this occurs, 2 H+ ions are released into the intermembrane space, contributing to the proton gradient. CoQH2 is now oxidized to ubiquinone and dissociates from the complex. The CoQ bound at the second site enters a transitional CoQH- radical state from accepting one of the electrons.

The second step of the cycle involves a repeat of the first: a new CoQH2 binds to the first site and transfers two electrons like before (and 2 more H+ ions released). Again, one electron passes to cytochrome c and one to cytochrome b, which this time works to reduce CoQH- to CoQH2 before it dissociates from complex III and can be recycled. In this way, one full cycle appears as follows:

  - 2 CoQH2(site 1) + CoQ(site 2) + 2 Cyt c(ox) + 2 H+(matrix) -> 2 CoQ(site 1) + CoQH2(site 2) + 2 Cyt c(red) + 4 H+(intermembrane)

  - **Complex IV**, also known as cytochrome c oxidase, oxidizes cytochrome c and transfers the electrons to oxygen, the final electron carrier in aerobic cellular respiration. The cytochrome proteins a and a3, in addition to heme and copper groups in complex IV transfer the donated electrons to the bound dioxygen species, converting it into molecules of water. The free energy

from the electron transfer causes 4 protons to move into the intermembrane space contributing to the proton gradient. Oxygen reduces via the following reaction:

- 2 cytochrome c(red) + ½O2 + 4 H+(matrix) -> 2 cytochrome c(ox) + 1 H2O + 2 H+(intermembrane)

ATP synthase, also called complex V, uses the ETC generated proton gradient across the inner mitochondrial membrane to form ATP. ATP-synthase contains up of F0 and F1 subunits, which act as a rotational motor system. F0 is hydrophobic and embedded in the inner mitochondrial membrane. It contains a proton corridor that is protonated and deprotonated repeatedly as H+ ions flow down the gradient from intermembrane space to matrix. The alternating ionization of F0 causes rotation, which alters the orientation of the F1 subunits. F1 is hydrophilic and faces the mitochondrial matrix. Conformational changes in F1 subunits catalyze the formation of ATP from ADP and Pi. For every 4 H+ ions, 1 ATP is produced. ATP-synthase can also be forced to run in reverse, consuming ATP to produce a hydrogen gradient, as is seen in some bacteria.

Molecular Level

Nicotinamide adenine dinucleotide has two forms: NAD+ (oxidized) and NADH (reduced). It is a dinucleotide connected by phosphate groups. One nucleoside has an adenine base and the other nicotinamide. When involved in metabolic redox reactions, the mechanism is as shown in Reaction 1.

- Reaction 1: RH2 + NAD+ -> R + H+ + NADH

R is the reactant, for example, sugar.

NADH enters the ETC at complex I and produces a total of 10 H+ ions through the ETC (4 from complex I, 4 from complex III, and 2 from complex IV). ATP-synthase synthesizes 1 ATP for 4 H+ ions. Therefore, 1 NADH = 10 H+, and 10/4 H+ per ATP = 2.5 ATP per NADH (**some sources round up**). When NADH is oxidized, it breaks into NAD+, H+, and 2 e- as shown in Reaction 2.

- Reaction 2: NADH -> H+ + NAD+ + 2 e-

Flavin adenine dinucleotide has 4 redox states, 3 of them being FAD (quinone, fully oxidized form), FADH- (semiquinone, partially oxidized), and FADH2 (hydroquinone, fully reduced). FAD is made up of an adenine nucleotide and a flavin mononucleotide (FMN), connected by phosphate groups. FMN is synthesized in part from vitamin B2 (riboflavin). FAD contains a highly stable aromatic ring, and FADH2 does not. When FADH2 oxidizes, it becomes aromatic and releases

energy, as seen in Reaction 3. This state makes FAD a potent oxidizing agent, with an even more positive reduction potential than NAD. FADH2 enters the ETC at complex II and creates a total of 1.5 ATP (4 H+ from complex III, and 2 H+ from complex IV; 6/4 H+ per ATP = 1.5 ATP per FADH2 **some sources round up**).

- Reaction 3: FADH2 -> FAD + 2 H+ + 2 e-

FAD also functions in several metabolic pathways outside of the ETC, including DNA repair (MTHF repair of UV damage), fatty acid beta-oxidation (acyl-CoA dehydrogenase), and synthesis of coenzymes (CoA, CoQ, heme).

Clinical Significance

Uncoupling Agents

An uncoupling agent dissociates the electron transport chain from phosphorylation by ATP-synthase, preventing the formation of ATP. Disruption of the phospholipid bilayer of membranes causes a fluid-like and disorganized state, which allows protons to flow through more freely. This proton leak weakens the electrochemical gradient, while also transferring protons without the use of ATP-synthase such that no ATP is produced.

While the cell becomes starved of ATP, the ETC will overwork in an attempt to shuttle more and more electrons to ATP-synthase without success. The ETC regularly produces heat as the electrons transfer from one carrier to the next, and this overactivity will raise the body temperature as a result. Additionally, cells will adapt to utilizing fermentation as if in anaerobic conditions; this may cause a type B lactic acidosis in affected patients.

Aspirin (Salicylic Acid)

- Salicylic acid is an uncoupler. Unique to salicylate poisoning, however, are signs of tinnitus and early respiratory alkalosis, which transitions to a mixed metabolic acidosis and respiratory alkalosis as the process progresses. Early treatment involves activated charcoal if presenting within 1 hour of ingestion, or sodium bicarbonate otherwise.

Thermogenin

- Thermogenin, also known as uncoupling protein 1 (UCP1), is found in brown adipose tissue. Brown adipose tissue has many small lipid droplets and a high concentration of mitochondria (which provide the "brown" color), in contrast to white adipose tissue, which has a single droplet.

This difference supports that brown fat is classically abundantly present in hibernating animals or newborns, who have delayed neurologic thermoregulation (ex. shivering) and are therefore at risk for hypothermia. These brown fat mitochondria contain more thermogenin than other cells, allowing for increased inner mitochondrial membrane disruption and proton leakage.

Oxidative Phosphorylation Inhibitors

Certain poisons can inhibit cellular oxidative phosphorylation such as rotenone, carboxin, antimycin A, cyanide, carbon monoxide (CO), sodium azide, and oligomycin. Rotenone inhibits complex I, carboxin inhibits complex II, antimycin A inhibits complex III, and cyanide and CO inhibit complex IV. Oligomycin inhibits ATP synthase.

Rotenone (and some barbiturates) – inhibits complex I (coenzyme Q binding site)

- Rotenone is a broadly used pesticide, but more often in the US as a piscicide (fish). Rotenone blocks complex I from passing electrons from the Fe-S clusters to ubiquinone. It is poorly absorbed through the skin, but rarely deadly as poisoning can cause vomiting and removal of the substance. However, purposeful ingestion can be fatal.

Carboxin – inhibits complex II (coenzyme Q binding site)

- Carboxin is a fungicide that is no longer in use because of newer, more broad-spectrum agents. Similar to rotenone, carboxin interferes with ubiquinone at the binding site.

Doxorubicin – coenzyme Q (theoretical)

- Doxorubicin is used in cancer chemotherapy, typically breast and bladder carcinomas, and lymphoma. A well-known side effect of doxorubicin is dilated cardiomyopathy. One proposed mechanism of causation is the generation of reactive oxygen species within myocardial tissue as the drug interferes with electron transfer by coenzyme Q.

Antimycin A – inhibits complex III (cytochrome c reductase)

- Antimycin A is a piscicide that binds to cytochrome c reductase at the Qi binding site. This activity prevents ubiquinone from binding and accepting an electron, thereby blocking the recycling of ubiquinol (CoQH2) by the Q cycle.

Carbon Monoxide (CO) – inhibits complex IV (cytochrome c oxidase)

- Carbon monoxide binds to and inhibits cytochrome c oxidase (complex IV). In addition to the disruption of the ETC, carbon monoxide also binds to hemoglobin at an oxygen-binding site converting it to carboxyhemoglobin. In this state, oxygen is displaced from hemoglobin, effectively blocking delivery to body tissues. The cardiac and central nervous systems, both organ systems which are highly dependent on oxygen consumption, manifest the common signs of CO poisoning. Symptoms such as tachycardia, hypotension, or arrhythmias may couple with fatigue, headache, nausea, vomiting, and changes in vision. More serious cases may display seizure, coma, retinal hemorrhages, or a characteristic cherry-red blood hue of the skin, though more often useful on autopsy (caution is critical: some patients may appear "normal" rather than pale/dusky because of inadequate tissue oxygenation).
- Sources of CO are paint strippers, house fires, wood-burning stoves, automobile exhaust, and other gasoline- or propane-fueled equipment. A CO saturation monitor can detect CO levels. Ratios of carboxyhemoglobin to hemoglobin greater than 10% are likely to show as symptomatic. Regular pulse oximetry devices read the percent of bound hemoglobin, irrespective of what is bound. Therefore, when CO is bound rather than O2, a patient's pulse Ox may still appear normal and cannot be used reliably. Instead, a co-oximeter should be used. Treatment for CO poisoning is to dissociate the bound CO with O2. Providing 100% supplemental oxygen via non-rebreather or administering hyperbaric oxygen are options.

Cyanide (CN) – inhibits complex IV (cytochrome c oxidase)

- Cyanide also binds to and inhibits cytochrome c oxidase (complex IV). Similar symptoms as a result of tissue hypoxia can present in affected patients. In contrast, these patients tend to have hypoxia that is not responsive to supplemental O2 and an almond breath odor. Typical sources of cyanide include house fires (furniture or rugs), jewelry cleaning solutions, plastic or rubber manufacturing, iatrogenic from prescribed nitroprusside, or even some fruit seeds (apricots, peaches, apples).
- Treatment can include nitrites to oxidize hemoglobin iron from Fe2+ to Fe3+, also known as methemoglobin, a conformation that binds cyanide, preventing it from contacting the ETC. However, this prevents blood cells from transporting oxygen, therefore requiring further treatment with methylene blue to reduce Fe3+ back to Fe2+. Another option is administering hydroxocobalamin, a form of vitamin B12, or thiosulfate, although thiosulfate is not time efficient and typically requires combination therapy with nitrites.

Oligomycin – inhibits ATP-synthase (complex V)

- Oligomycin is a macrolide antibiotic synthesized by Streptomyces species that inhibits the F0 subunit of ATP-synthase, preventing ATP production. Its predominant use is for research purposes.

Anaerobic respiration: Anaerobic respiration is the formation of ATP without oxygen. This method still incorporates the respiratory electron transport chain, but without using oxygen as the terminal electron acceptor. Instead, molecules such as sulfate (SO_4^{2-}), nitrate (NO_3^-), or sulfur (S) are used as electron acceptors. These molecules have a lower reduction potential than oxygen; thus, less energy is formed per molecule of glucose in anaerobic versus aerobic conditions.

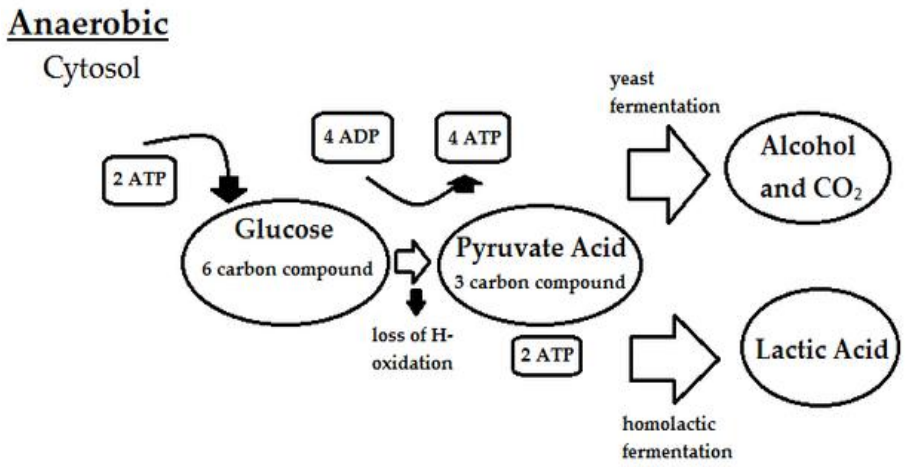


Figure: **Anaerobic Respiration**: A molecule other than oxygen is used as the terminal electron acceptor in anaerobic respiration.

Nitrate: Many different types of electron acceptors may be used for anaerobic respiration. Denitrification is the utilization of nitrate (NO_3^-) as the terminal electron acceptor. Nitrate, like oxygen, has a high reduction potential. This process is widespread, and used by many members of Proteobacteria. Many denitrifying bacteria can also use ferric iron (Fe^{3+}) and different organic electron acceptors.

Sulfate reduction uses sulfate (SO_2^{-4}) as the electron acceptor, producing hydrogen sulfide (H_2S) as a metabolic end product. Sulfate reduction is a relatively energetically poor process, and is used by many Gram negative bacteria found within the δ-Proteobacteria. It is also used in Gram-positive organisms related to Desulfotomaculum or the archaeon Archaeoglobus.

Sulfate reduction requires the use of electron donors, such as the carbon compounds lactate and pyruvate (organotrophic reducers), or hydrogen gas (lithotrophic reducers). Some unusual autotrophic sulfate-reducing bacteria, such as *Desulfotignum phosphitoxidans*, can use phosphite (HPO_3^-) as an electron donor. Others, such as certain *Desulfovibrio* species, are capable of sulfur disproportionation (splitting one compound into an electron donor and an electron acceptor) using elemental sulfur (S0), sulfite (SO_3^{-2}), and thiosulfate ($S_2O_3^{2-}$) to produce both hydrogen sulfide (H_2S) and sulfate (SO^{2-}).

Acetogenesis is a type of microbial metabolism that uses hydrogen (H_2) as an electron donor and carbon dioxide (CO_2) as an electron acceptor to produce acetate, the same electron donors and acceptors used in methanogenesis.

Ferric iron (Fe^{3+}) is a widespread anaerobic terminal electron acceptor used by both autotrophic and heterotrophic organisms. Electron flow in these organisms is similar to those in electron transport, ending in oxygen or nitrate, except that in ferric iron-reducing organisms the final enzyme in this system is a ferric iron reductase. Since some ferric iron-reducing bacteria (*e.g.G. metallireducens*) can use toxic hydrocarbons (*e.g.* toluene) as a carbon source, there is significant interest in using these organisms as bioremediation agents in ferric iron contaminated aquifers.

Other inorganic electron acceptors include the reduction of Manganic ion (Mn^{4+}) to manganous (Mn^{2+}), Selenate (SeO_4^{2-}) to selenite (SeO_3^{2-}) to selenium (Se), Arsenate (AsO_4^{3-}) to arsenite (AsO_3^{3-}), and Uranyl (UO_2^{2+}) to uranium dioxide (UO_2)

Organic compounds may also be used as electron acceptors in anaerobic respiration. These include the reduction of fumarate to succinate, Trimethylamine N-oxide (TMAO) to trimethylamine (TMA), and Dimethyl sulfoxide (DMSO) to Dimethyl sulfide (DMS).

In anaerobic respiration, denitrification utilizes nitrate (NO_3^-) as a terminal electron acceptor in the respiratory electron transport chain. Denitrification is a widely used process; many facultative anaerobes use denitrification because nitrate, like oxygen, has a high reduction potential

Denitrification is a microbially facilitated process involving the stepwise reduction of nitrate to nitrite (NO_2^-) nitric oxide (NO), nitrous oxide (N_2O), and, eventually, to dinitrogen (N_2) by the enzymes nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase. The complete denitrification process can be expressed as a redox reaction: $2\ NO^{3-} + 10\ e^- + 12\ H^+ \rightarrow N_2 + 6\ H_2O$.

Protons are transported across the membrane by the initial NADH reductase, quinones and nitrous oxide reductase to produce the electrochemical gradient critical for respiration. Some organisms (e.g. *E. coli*) only produce nitrate reductase and therefore can accomplish only the first reduction leading to the accumulation of nitrite. Others (e.g. *Paracoccus denitrificans* or *Pseudomonas stutzeri*) reduce nitrate completely. Complete denitrification is an environmentally significant process because some intermediates of denitrification (nitric oxide and nitrous oxide) are significant greenhouse gases that react with sunlight and ozone to produce nitric acid, a component of acid rain. Denitrification is also important in biological wastewater treatment, where it can be used to reduce the amount of nitrogen released into the environment, thereby reducing eutrophication.

Denitrification takes place under special conditions in both terrestrial and marine ecosystems. In general, it occurs where oxygen is depleted and bacteria respire nitrate as a substitute terminal electron acceptor. Due to the high concentration of oxygen in our atmosphere, denitrification only takes place in anaerobic environments where oxygen consumption exceeds the oxygen supply and where sufficient quantities of nitrate are present. These environments may include certain soils and groundwater, wetlands, oil reservoirs, poorly ventilated corners of the ocean, and in sea floor sediments.

Denitrification is performed primarily by heterotrophic bacteria (e.g. *Paracoccus denitrificans*), although autotrophic denitrifiers have also been identified (e.g., *Thiobacillus denitrificans*). Generally, several species of bacteria are involved in the complete reduction of nitrate to molecular nitrogen, and more than one enzymatic pathway have been identified in the reduction process.

Rhizobia are soil bacteria with the unique ability to establish a N_2-fixing symbiosis on legume roots. When faced with a shortage of oxygen, some *rhizobia* species are able to switch from O_2-respiration to using nitrates to support respiration.

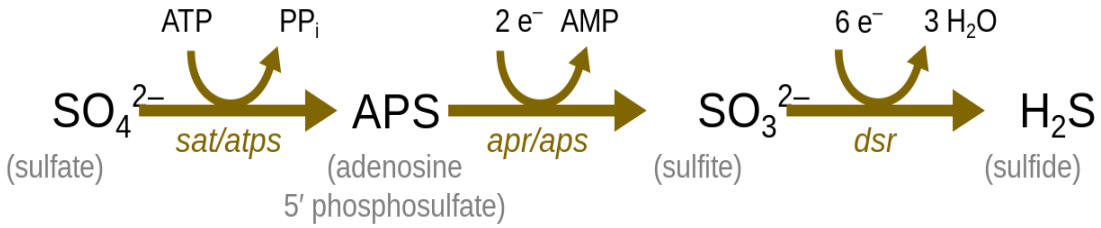
The direct reduction of nitrate to ammonium (dissimilatory nitrate reduction) can be performed by organisms with the nrf- gene. This is a less common method of nitrate reduction than denitrification in most ecosystems. Other genes involved in denitrification include nir (nitrite reductase) and nos (nitrous oxide reductase), which are possessed by such organisms as *Alcaligenes faecalis*, *Alcaligenes xylosoxidans*, *Pseudomonas spp*, *Bradyrhizobium japonicum*, and *Blastobacter denitrificans*.

Sulfate reduction is a type of anaerobic respiration that utilizes sulfate as a terminal electron acceptor in the electron transport chain. Compared to aerobic respiration, sulfate reduction is a relatively energetically poor process, though it is a vital mechanism for bacteria and archaea living in oxygen-depleted, sulfate-rich environments.

Many sulfate reducers are organotrophic, using carbon compounds, such as lactate and pyruvate (among many others) as electron donors, while others are lithotrophic, and use hydrogen gas (H_2) as an electron donor. Some unusual autotrophic sulfate-reducing bacteria (e.g., *Desulfotignum phosphitoxidans*) can use phosphite (HPO^{3-}) as an electron donor, whereas others (e.g., *Desulfovibrio sulfodismutans*, *Desulfocapsa thiozymogenes*, and *Desulfocapsa sulfoexigens*) are capable of sulfur disproportionation (splitting one compound into two different compounds, in this case an electron donor and an electron acceptor) using elemental sulfur (S0), sulfite (SO_3^{2-}), and thiosulfate ($S_2O_3^{2-}$) to produce both hydrogen sulfide (H_2S) and sulfate (SO_4^{2-}).

Before sulfate can be used as an electron acceptor, it must be activated. This is done by the enzyme ATP-sulfurylase, which uses ATP and sulfate to create adenosine 5′-phosphosulfate (APS). APS is subsequently reduced to sulfite and AMP. Sulfite is then further reduced to sulfide, while AMP is turned into ADP using another molecule of ATP. The overall process, thus, involves an investment of two molecules of the energy carrier ATP, which must to be regained from the reduction.

All sulfate-reducing organisms are strict anaerobes. Because sulfate is energetically stable, it must be activated by adenylation to form APS (adenosine 5′-phosphosulfate) to form APS before it can be metabolized, thereby consuming ATP. The APS is then reduced by the enzyme APS reductase to form sulfite (SO_3^{2-}) and AMP. In organisms that use carbon compounds as electron donors, the ATP consumed is accounted for by fermentation of the carbon substrate. The hydrogen produced during fermentation is actually what drives respiration during sulfate reduction.



Dissimilatory sulfate reduction

Sulfate-reducing bacteria can be traced back to 3.5 billion years ago and are considered to be among the oldest forms of microorganisms, having contributed to the sulfur cycle soon after life emerged on Earth. Sulfate-reducing bacteria are common in anaerobic environments (such as seawater, sediment, and water rich in decaying organic material) where they aid in the degradation of organic materials. In these anaerobic environments, fermenting bacteria extract energy from large organic molecules; the resulting smaller compounds (such as organic acids and alcohols) are further oxidized by acetogens, methanogens, and the competing sulfate-reducing bacteria.

Many bacteria reduce small amounts of sulfates in order to synthesize sulfur-containing cell components; this is known as assimilatory sulfate reduction. By contrast, sulfate-reducing bacteria reduce sulfate in large amounts to obtain energy and expel the resulting sulfide as waste; this is known as "dissimilatory sulfate reduction." Most sulfate-reducing bacteria can also reduce other oxidized inorganic sulfur compounds, such as sulfite, thiosulfate, or elemental sulfur (which is reduced to sulfide as hydrogen sulfide).

Toxic hydrogen sulfide is one waste product of sulfate-reducing bacteria; its rotten egg odor is often a marker for the presence of sulfate-reducing bacteria in nature. Sulfate-reducing bacteria are responsible for the sulfurous odors of salt marshes and mud flats. Much of the hydrogen sulfide will react with metal ions in the water to produce metal sulfides. These metal sulfides, such as ferrous sulfide (FeS), are insoluble and often black or brown, leading to the dark color of sludge. Thus, the black color of sludge on a pond is due to metal sulfides that result from the action of sulfate-reducing bacteria.

Figure: **Black sludge**: The black color of this pond is due to metal sulfides that result from the action of sulfate-reducing bacteria.

Some sulfate-reducing bacteria play a role in the anaerobic oxidation of methane (CH_4+ $SO4^{2-} \rightarrow HCO_3– + HS– + H_2O$). An important fraction of the methane formed by methanogens below the seabed is oxidized by sulfate-reducing bacteria in the transition zone separating the methanogenesis from the sulfate reduction activity in the sediments.This process is also considered a major sink for sulfate in marine sediments. In hydrofracturing fluids used to frack shale formations to recover methane (shale gas), biocide compounds are often added to water to inhibit the microbial activity of sulfate-reducing bacteria in order to avoid anaerobic methane oxidation and to minimize potential production loss.

Sulfate-reducing bacteria often create problems when metal structures are exposed to sulfate-containing water. The interaction of water and metal creates a layer of molecular hydrogen on the metal surface. Sulfate-reducing bacteria oxidize this hydrogen, creating hydrogen sulfide, which contributes to corrosion. Hydrogen sulfide from sulfate-reducing bacteria also plays a role in the biogenic sulfide corrosion of concrete, and sours crude oil.

Sulfate-reducing bacteria may be utilized for cleaning up contaminated soils; some species are able to reduce hydrocarbons, such as benzene, toluene, ethylbenzene, and xylene. Sulfate-reducing bacteria may also be a way to deal with acid mine waters.

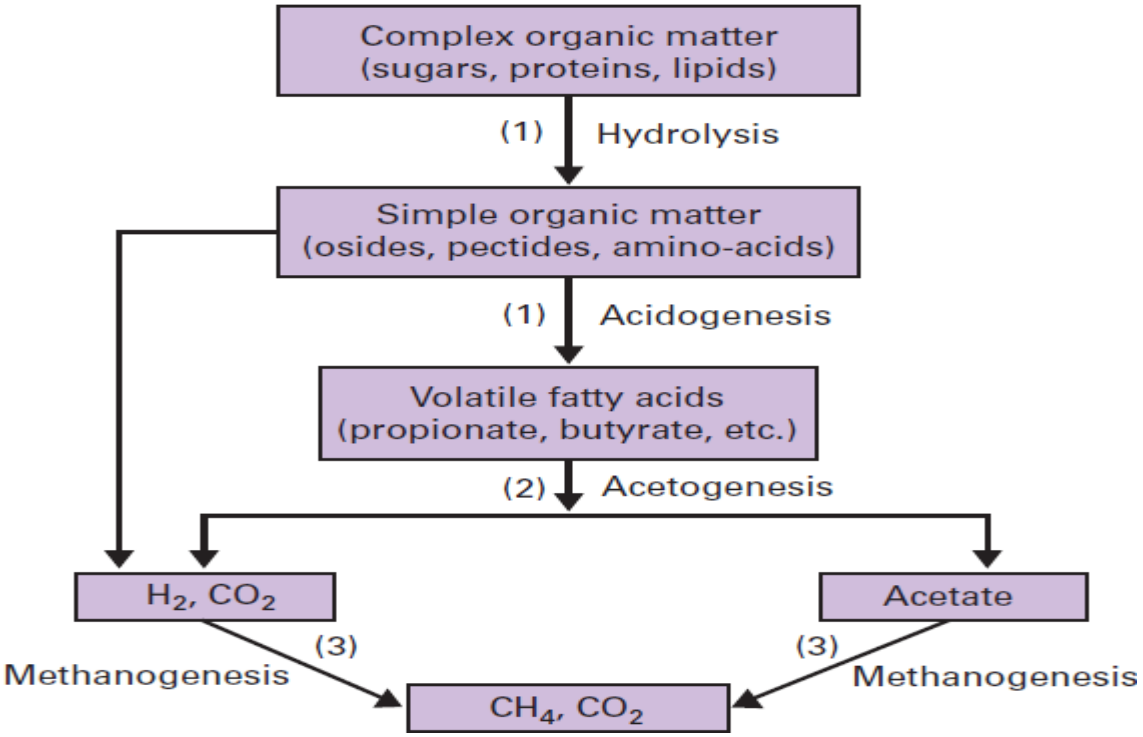
Methanogenesis, or biomethanation, is a form of anaerobic respiration that uses carbon as the terminal electron acceptor, resulting in the production of methane. The carbon is sourced from a small number of low molecular weight organic compounds, such as carbon dioxide, acetic acid, formic acid (formate), methanol, methylamines, dimethyl sulfide, and methanethiol. The two best described pathways of methanogenesis use carbon dioxide or acetic acid as the terminal electron acceptor:

$CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O$

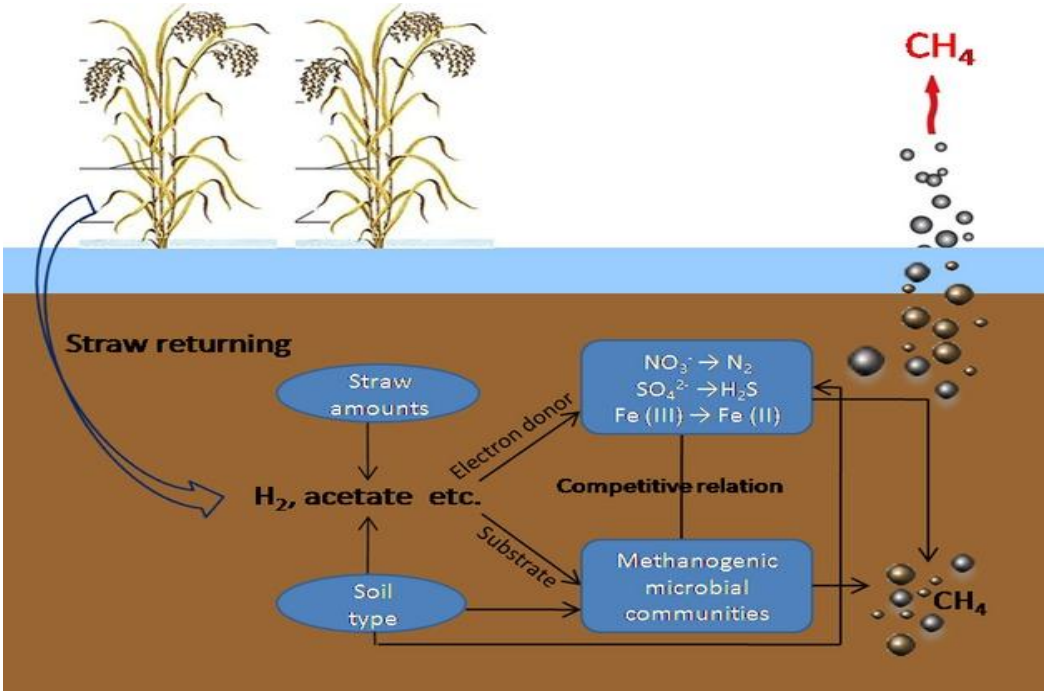
$CH_3COOH \rightarrow CH_4 + CO_2$

The biochemistry of methanogenesis is relatively complex. It involves the coenzymes and cofactors F420, coenzyme B, coenzyme M, methanofuran, and methanopterin.

Microbes capable of producing methane are called methanogens. They have been identified only from the domain Archaea – a group that is phylogenetically distinct from eukaryotes and bacteria – though many live in close association with anaerobic bacteria. The production of methane is an important and widespread form of microbial metabolism, and in most environments, it is the final step in the decomposition of biomass.



Methanogenesis biochemistry and microbiology



Methane production and methanogenic archaeal communities in two types of paddy soil amended with different amounts of rice straw

During the decay process, electron acceptors (such as oxygen, ferric iron, sulfate, and nitrate) become depleted, while hydrogen (H2), carbon dioxide, and light organics produced by fermentation accumulate. During advanced stages of organic decay, all electron acceptors become depleted except carbon dioxide, which is a product of most catabolic processes. It is not depleted like other potential electron acceptors.

Only methanogenesis and fermentation can occur in the absence of electron acceptors other than carbon. Fermentation only allows the breakdown of larger organic compounds, and produces small organic compounds. Methanogenesis effectively removes the semi-final products of decay: hydrogen, small organics, and carbon dioxide. Without methanogenesis, a great deal of carbon (in the form of fermentation products) would accumulate in anaerobic environments.

Methanogenesis also occurs in the guts of humans and other animals, especially ruminants. In the rumen, anaerobic organisms, including methanogens, digest cellulose into forms usable by the animal. Without these microorganisms, animals such as cattle would not be able to consume grass. The useful products of methanogenesis are absorbed by the gut. Methane is released from the animal mainly by belching (eructation). The average cow emits around 250 liters of methane per day. Some, but not all, humans emit methane in their flatus!

Some experiments even suggest that leaf tissues of living plants emit methane, although other research indicates that the plants themselves do not actually generate methane; they are just absorbing methane from the soil and then emitting it through their leaf tissues. There may still be some unknown mechanism by which plants produce methane, but that is by no means certain.

Methane is one of the earth’s most important greenhouse gases, with a global warming potential 25 times greater than carbon dioxide (averaged over 100 years). Therefore, the methane produced by methanogenesis in livestock is a considerable contributor to global warming.

Methanogenesis can also be beneficially exploited. It is the primary pathway that breaks down organic matter in landfills (which can release large volumes of methane into the atmosphere if left uncontrolled), and can be used to treat organic waste and to produce useful compounds. Biogenic methane can be collected and used as a sustainable alternative to fossil fuels.

Fermentation

The production of energy requires oxygen. The electron transport chain, where the majority of ATP is formed, requires a large input of oxygen. However, many organisms have developed strategies to carry out metabolism without oxygen, or can switch from aerobic to anaerobic cell respiration when oxygen is scarce.

Figure: ***Anaerobic bacteria****: The green color seen in these coastal waters is from an eruption of hydrogen sulfide-producing bacteria. These anaerobic, sulfate-reducing bacteria release hydrogen sulfide gas as they decompose algae in the water.*

During cellular respiration, some living systems use an organic molecule as the final electron acceptor. Processes that use an organic molecule to regenerate NAD^+ from NADH are collectively referred to as fermentation. In contrast, some living systems use an inorganic molecule as a final electron acceptor. Both methods are called anaerobic cellular respiration, where organisms convert energy for their use in the absence of oxygen.

Certain prokaryotes, including some species of bacteria and archaea, use anaerobic respiration. For example, the group of archaea called methanogens reduces carbon dioxide to methane to oxidize NADH. These microorganisms are found in soil and in the digestive tracts of ruminants, such as cows and sheep. Similarly, sulfate-reducing bacteria and archaea, most of which are anaerobic, reduce sulfate to hydrogen sulfide to regenerate NAD^+ from NADH.

Eukaryotes can also undergo anaerobic respiration. Some examples include alcohol fermentation in yeast and lactic acid fermentation in mammals.

Lactic Acid Fermentation

The fermentation method used by animals and certain bacteria (like those in yogurt) is called lactic acid fermentation. This type of fermentation is used routinely in mammalian red blood cells and in skeletal muscle that has an insufficient oxygen supply to allow aerobic respiration to continue (that is, in muscles used to the point of fatigue). The excess amount of lactate in those muscles is what causes the burning sensation in your legs while running. This pain is a signal to rest the overworked muscles so they can recover. In these muscles, lactic acid accumulation must be removed by the blood circulation and the lactate brought to the liver for further metabolism. The chemical reactions of lactic acid fermentation are the following:

Pyruvic acid + NADH ↔ lactic acid + NAD^+

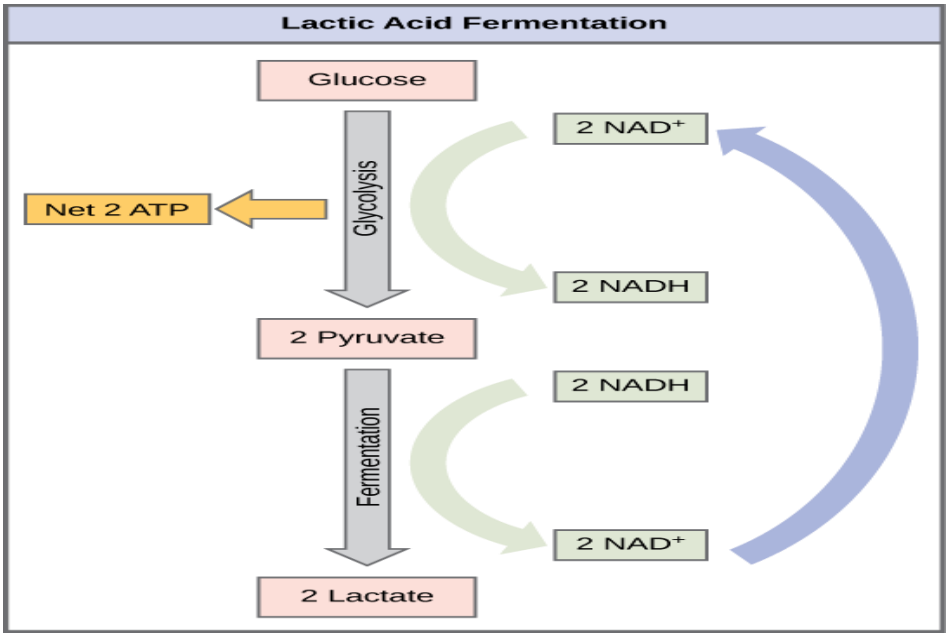


Figure: ***Lactic acid fermentation****: Lactic acid fermentation is common in muscle cells that have run out of oxygen.*

The enzyme used in this reaction is lactate dehydrogenase (LDH). The reaction can proceed in either direction, but the reaction from left to right is inhibited by acidic conditions. Such lactic acid accumulation was once believed to cause muscle stiffness, fatigue, and soreness, although more recent research disputes this hypothesis. Once the lactic acid has been

removed from the muscle and circulated to the liver, it can be reconverted into pyruvic acid and further catabolized for energy.

Alcohol Fermentation

Another familiar fermentation process is alcohol fermentation, which produces ethanol, an alcohol. The use of alcohol fermentation can be traced back in history for thousands of years. The chemical reactions of alcoholic fermentation are the following (Note: CO_2 does not participate in the second reaction):

Pyruvic acid → CO2 + acetaldehyde + NADH → ethanol + NAD^+

Figure: ***Alcohol Fermentation****: Fermentation of grape juice into wine produces CO2 as a byproduct. Fermentation tanks have valves so that the pressure inside the tanks created by the carbon dioxide produced can be released.*

The first reaction is catalyzed by pyruvate decarboxylase, a cytoplasmic enzyme, with a coenzyme of thiamine pyrophosphate (TPP, derived from vitamin B_1 and also called thiamine). A carboxyl group is removed from pyruvic acid, releasing carbon dioxide as a gas. The loss of carbon dioxide reduces the size of the molecule by one carbon, making acetaldehyde. The second reaction is catalyzed by alcohol dehydrogenase to oxidize NADH to NAD^+ and reduce acetaldehyde to ethanol.

The fermentation of pyruvic acid by yeast produces the ethanol found in alcoholic beverages. Ethanol tolerance of yeast is variable, ranging from about 5 percent to 21 percent, depending on the yeast strain and environmental conditions.

Other Types of Fermentation

Various methods of fermentation are used by assorted organisms to ensure an adequate supply of NAD^+ for the sixth step in glycolysis. Without these pathways, that step would not occur and no ATP would be harvested from the breakdown of glucose.Other fermentation methods also occur in bacteria. Many prokaryotes are facultatively anaerobic. This means that they can switch between aerobic respiration and fermentation, depending on the availability of oxygen. Certain prokaryotes, like *Clostridia*, are obligate anaerobes. Obligate anaerobes live and grow in the absence of molecular oxygen. Oxygen is a poison to these microorganisms, killing them on exposure.

It should be noted that all forms of fermentation, except lactic acid fermentation, produce gas. The production of particular types of gas is used as an indicator of the fermentation of specific carbohydrates, which plays a role in the laboratory identification of the bacteria.

Formate formation

Formate is produced by the cleavage of pyruvate. This reaction is catalysed by the enzyme pyruvate-formate lyase (PFL), which plays an important role in regulating anaerobic fermentation in *E. coli*.

pyruvate + CoA → acetyl-CoA + **formate**

Acetate formation

Pyruvate is converted into acetyl-coenzyme A (acetyl-CoA) by the enzyme pyruvate dehydrogenase. This acetyl-CoA is then converted into acetate in *E. coli*, whilst producing ATP by substrate-level phosphorylation. Acetate formation requires two enzymes: phosphate acetyltransferase and acetate kinase.

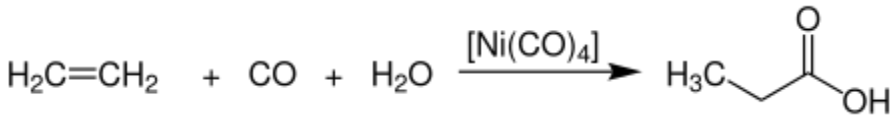
The mixed acid fermentation pathway is characteristic of the family Enterobacteriaceae, which includes *E. coli*

acetyl-CoA + phosphate → acetyl-phosphate + CoA

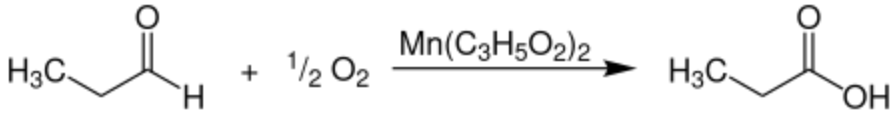
acetyl-phosphate + ADP → **acetate** + ATP

Propionate

In industry, propionic acid is mainly produced by the hydrocarboxylation of ethylene using nickel carbonyl as the catalyst



It is also produced by the aerobic oxidation of propionaldehyde. In the presence of cobalt or manganese salts (manganese propionate is most commonly used), this reaction proceeds rapidly at temperatures as mild as 40–50 °C:



Large amounts of propionic acid were once produced as a byproduct of acetic acid manufacture. At the current time, the world's largest producer of propionic acid is BASF, with approximately 150 kt/a production capacity.

Butane diol

2,3-Butanediol fermentation is anaerobic fermentation of glucose with 2,3-butanediol as one of the end products. The overall stoichiometry of the reaction is

2 pyruvate + NADH --> $2CO_2$ + 2,3-butanediol.

Butanediol fermentation is typical for the facultative anaerobes *Klebsiella* and *Enterobacter* and is tested for using the Voges–Proskauer (VP) test. There are other alternative strains that can be used, talked about in details in the Alternative Bacteria Strains section below.

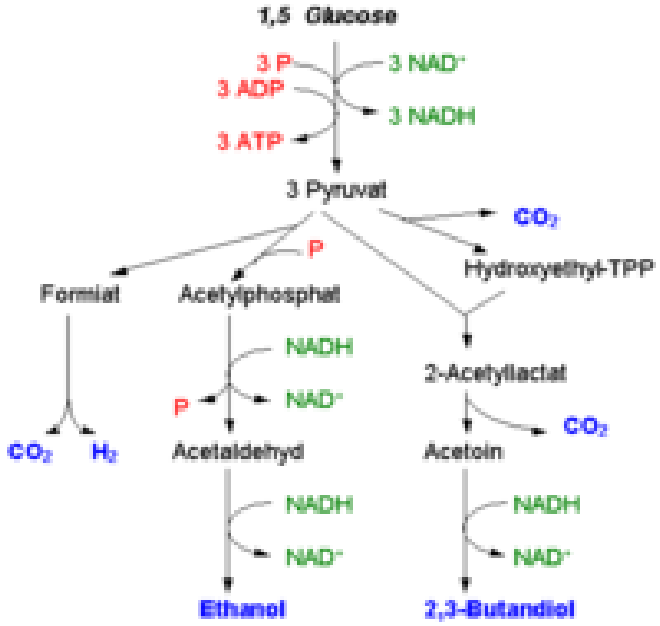


Figure 2: The fermentation process to produce butanediol

The metabolic function of 2,3-butanediol is not known, although some have speculated that it was an evolutionary advantage for these microorganisms to produce a neutral product that's less inhibitory than other partial oxidation products and doesn't reduce the pH as much as mixed acids.

There are many important industrial applications that butanediol can be used for, including antifreeze, food additives, antiseptic, and pharmaceuticals. It also is produced naturally in various places of the environment.

The Entner–Doudoroff pathway describes an alternate series of reactions that catabolize glucose to pyruvate using a set of enzymes different from those used in either glycolysis or the pentose phosphate pathway. Glycolysis (from glycose, an older term for glucose + -lysis degradation) is the metabolic pathway that converts glucose C6H12O6C6H12O6, into pyruvate, CH3COCOO−CH3COCOO−. The free energy released in this process is used to form the high-energy compounds ATP (adenosine triphosphate) and NADH (reduced nicotinamide adenine dinucleotide). Most bacteria use glycolysis and the pentose phosphate pathway. This pathway was first reported in 1952 by Michael Doudoroff and Nathan Entner.

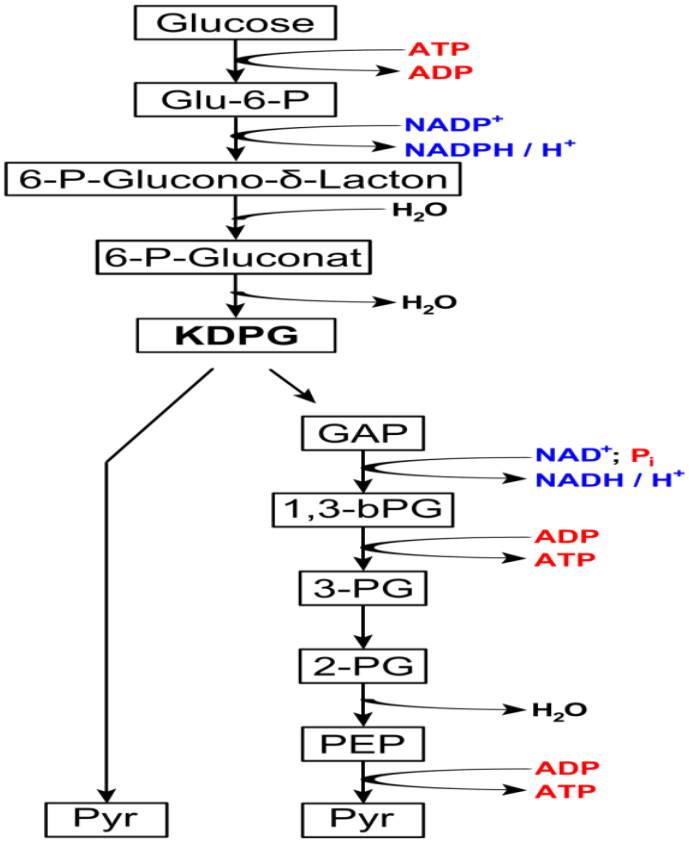


Figure: ***The Entner–Doudoroff Pathway****: This is a diagram of the Entner-Doudoroff pathway (KDPG: 2-keto-3-deoxy-6-phosphogluconate).*

Distinct features of the Entner–Doudoroff pathway are that it occurs only in prokaryotes and it uses 6-phosphogluconate dehydratase and 2-keto-3-deoxyphosphogluconate aldolase to create pyruvate from glucose. The Entner–Doudoroff pathway has a net yield of 1 ATP for every glucose molecule processed, as well as 1 NADH and 1 NADPH. By comparison, glycolysis has a net yield of 2 ATP and 2 NADH for every one glucose molecule processed.

There are a few bacteria that substitute classic glycolysis with the Entner-Doudoroff pathway. They may lack enzymes essential for glycolysis, such as phosphofructokinase-1. This pathway is generally found in *Pseudomonas*, *Rhizobium*, *Azotobacter*, *Agrobacterium*, and a few other Gram-negative genera. Very few Gram-positive bacteria have this pathway, with *Enterococcus faecalis* being a rare exception. Most organisms that use the pathway are aerobes due to the low ATP yield per glucose such as Pseudomonas, a genus of Gram-negative bacteria, and Azotobacter, a genus of Gram-negative bacteria.

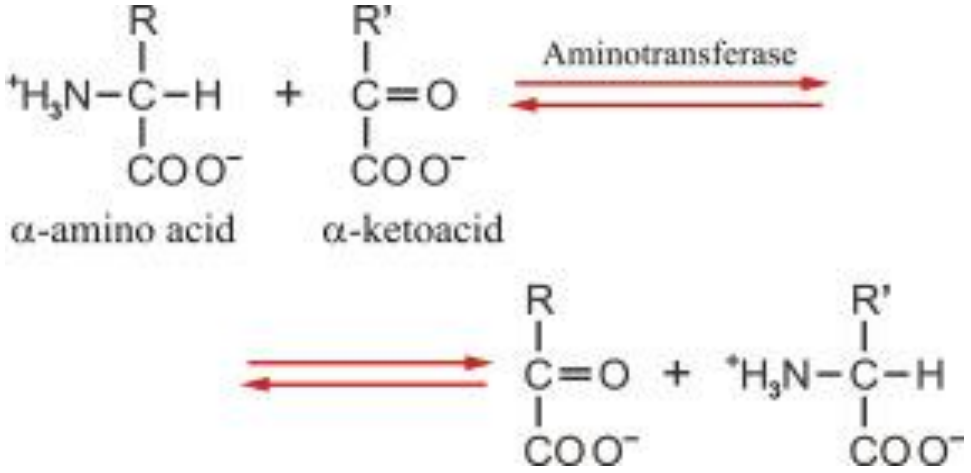
Amino acid metabolism

Peptidases are enzymes capable of cleaving, and thereby often inactivating, small peptides. They are widely distributed on the surface of many different cell types, with the catalytic site exposed only at the external surface. Peptidases are involved in a variety of processes, including peptide- mediated inflammatory responses, stromal cell- dependent B lymphopoiesis, and T- cell activation. In addition, some peptidases may have functions that are not based on their enzymatic activity.

Peptidases are classified according to the location of the cleavage site in the putative substrate. Endopeptidases recognize specific amino acids in the middle of the peptide, whereas exopeptidases recognize one or two terminal amino acids. Exopeptidases that attack peptides from the N- terminus (removing either single amino acids or a dipeptide) are termed (dipeptidyl) aminopeptidases, whereas peptidases attacking the C- terminus are termed carboxypeptidases.

Transamination This process refers to the transfer of the amino acid α-amine group to a α-keto acid. The amino acid becomes a keto acid, and the α-keto acid acceptor of the amine group is converted into the corresponding amino acid.

The general equation is:



This reaction is readily reversible; it is catalyzed by *transaminase* or *aminotransferase*, an enzyme that uses pyridoxal phosphate, a coenzyme that tightly binds to the transaminase.

Pyridoxal phosphate is derived from pyridoxine, a B complex vitamin. It participates in numerous reactions forming with the amino acid a Schiff base intermediate compound (Fig. 16.3). This reaction allows that all α carbon bonds in AA become more labile, facilitating subsequent reactions (transfer of the amine group, decarboxylation, and others). The enzyme

is responsible for guiding the direction of the reaction and ensuring the nature of the change. Aminotransferases catalyze the separation and transfer of the amine group attached to the α-carbon. Pyridoxal phosphate serves as an acceptor and transporter of the amine group.

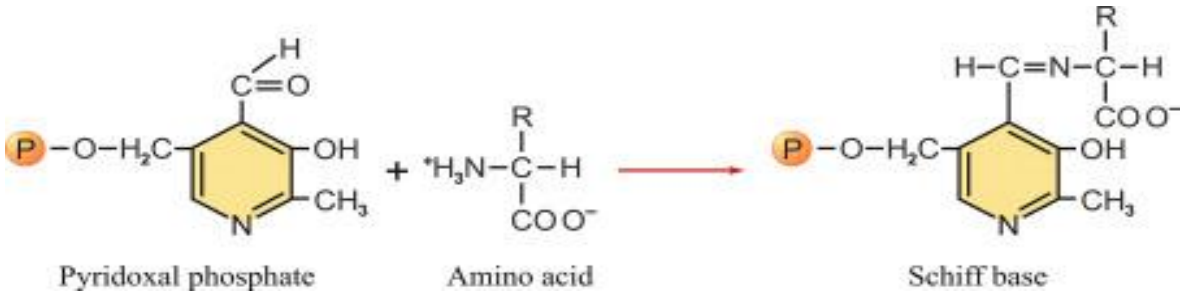
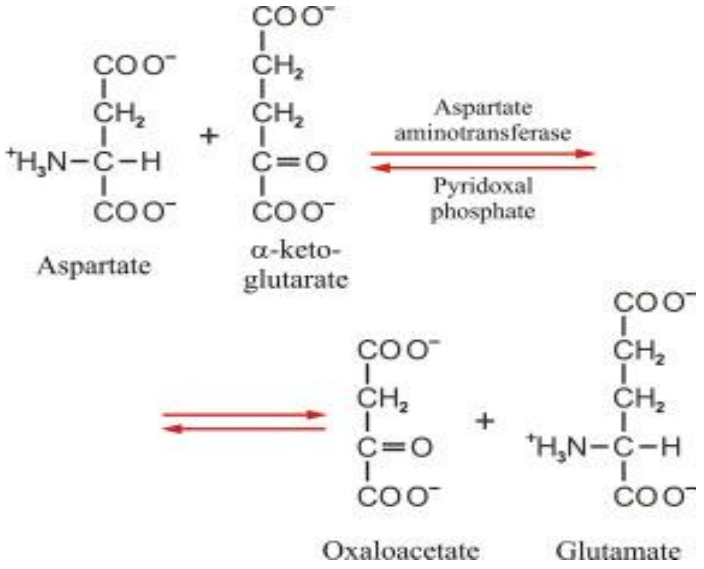


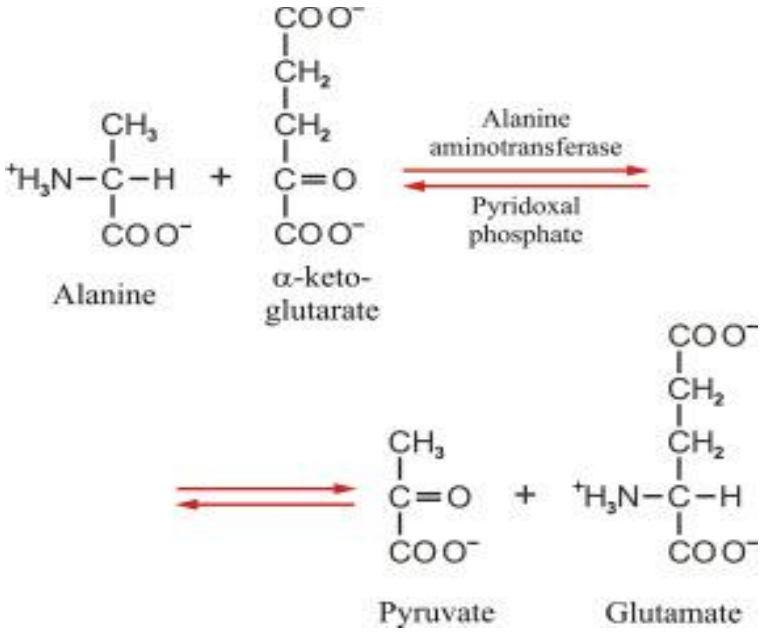
Figure. Schiff base formation (amino acid-pyridoxal phosphate).

Transamination is a bimolecular reaction and its mechanism is well known ("ping-pong" mechanism, or double-displacement reaction, characterized by the change of the enzyme into an intermediate form when the first substrate to product reaction occurs). First, the amino acid is bound to the active site to form a Schiff base with pyridoxal phosphate (the "ping" part of the reaction). Then, the α-amine group is separated by hydrolysis and an α-keto acid, derived from the original amino acid, is formed and released (the "pong" part of the reaction). The prosthetic group of the enzyme is converted into pyridoxamine phosphate. Subsequently, another α-keto acid enters the catalytic site as a second substrate, forming a Schiff base with pyridoxamine phosphate. The amine group is transferred to the keto acid, pyridoxal phosphate is regenerated, and the newly formed amino acid is released. Both substrates bind successively and independently to the enzyme and the first product is removed before the second substrate is bound. Pyridoxal phosphate acts as a transient acceptor of the amine group. Commonly, the α-keto acid is α-ketoglutarate; the enzyme receives its name from the amino acid donor of the amine group. For example, aspartate aminotransferase catalyzes the following reversible reaction:



This reaction is particularly important in the liver. In the reverse reaction, oxaloacetate acts as an acceptor of the amine group donated by glutamate.

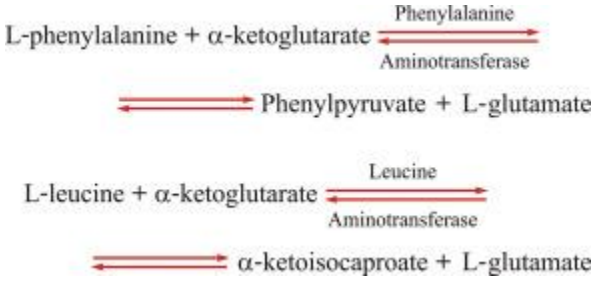
Alanine aminotransferase is responsible for the following reaction:



One of the substrates/products of this reaction, alanine, is an important amine carrier. In the muscle, the amine groups are transferred from AA other than α-ketoglutarate to produce glutamate and eventually pyruvate. Alanine, which enters the circulation, is taken up by tissues, mainly the liver, where it undergoes transamination again to regenerate glutamate and pyruvate.

Aspartate aminotransferase and alanine aminotransferase are the names recommended for these enzymes by the IUBMB; however, the initials GOT and GPT (glutamic-oxaloacetic and glutamic-pyruvic acid transaminases) are widely used in clinics. Both of these aminotransferases are particularly abundant in the liver and heart, which explains the increase of these enzymes in plasma when there are pathological processes of these organs (i.e., hepatitis and myocardial infarction). Hence, the determination of these enzymes in plasma is often used for diagnostic and prognostic purposes.

Other examples of transamination reactions include the following:



These reactions are reversible. Certain aminotransferases are expressed as two isozymes with different intracellular localization, the cytosol, and the mitochondrial matrix.

With the exception of lysine and threonine, all AA are involved in transamination reactions with the α-keto acids pyruvate, oxaloacetate, or α-ketoglutarate, which are converted to alanine, aspartate, or glutamate, respectively. The original AA form the corresponding α-keto acids. In turn, alanine and aspartate, produced by transamination from pyruvate, and oxaloacetate react with α-ketoglutarate. The amine groups are used for the formation of glutamate (Fig. 16.4).

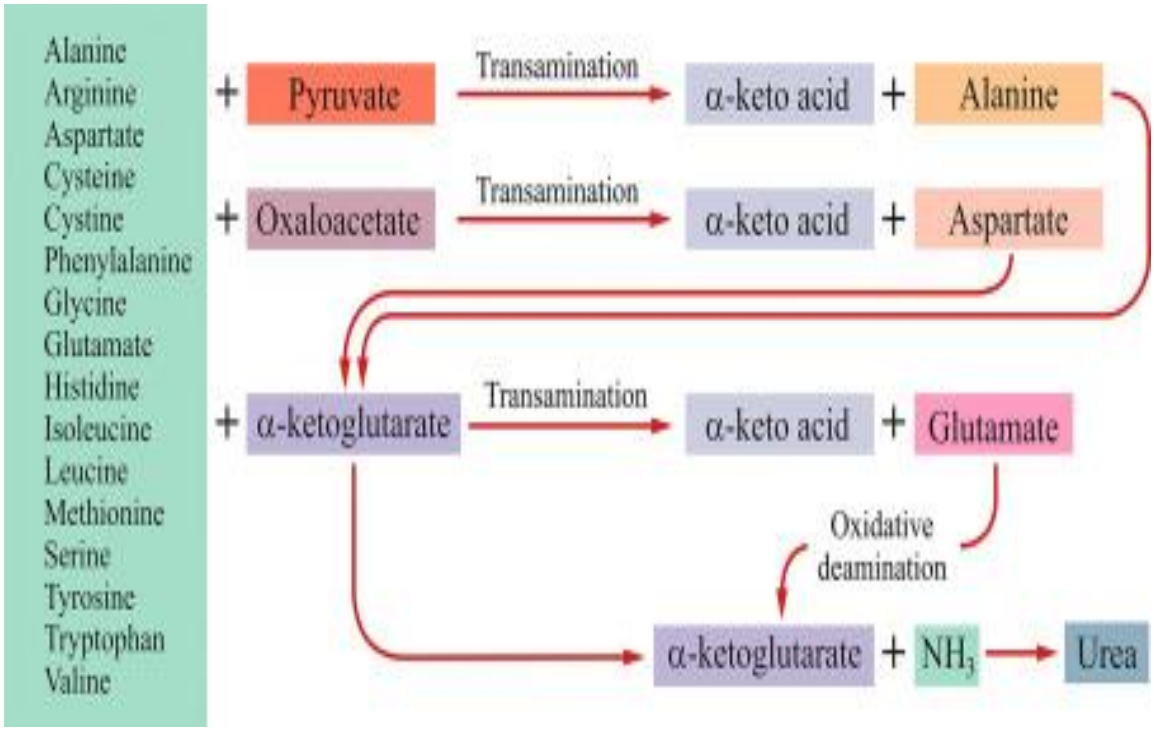
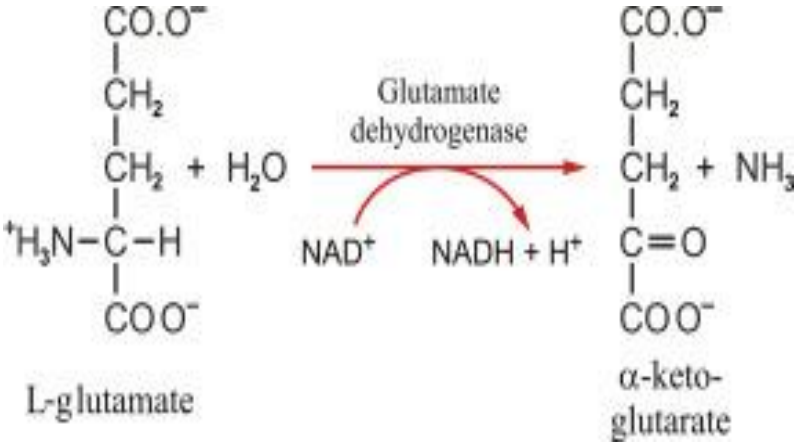


Figure. Fate of the amine group of amino acids.

In transamination reactions, the amine group of the amino acid is not eliminated but transferred to a keto acid to form another amino acid. For this reason, the reaction is not only the first step in the degradation of the AA carbon chain but also the last step in the synthesis of AA. Through transamination, a given amino acid can be generated if the corresponding α-keto acid is available.

Glutamate deamination

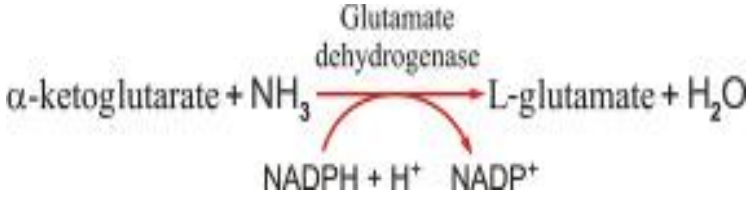
The substrate most frequently involved in transamination reactions is α-ketoglutarate. Amine groups from almost all AA converge to form glutamate. The nitrogen group of glutamate can be removed by oxidative deamination catalyzed by *glutamate dehydrogenase*. This enzyme, active in most mammalian tissues, uses NAD and NADP as coenzymes. In the forward reaction, NAD^+ usually participates and α-ketoglutarate and ammonia are formed.



Most of the ammonia produced in tissues is generated by this reaction. At physiological pH, ammonia (NH_3) captures a proton and becomes ammonium ion (NH_4^+).

Glutamate dehydrogenase is found in the mitochondrial matrix. It is an allosteric enzyme, activated by ADP and GDP and inhibited by ATP and GTP. When the ADP level in the cell is high, the enzyme is activated. Increased production of α-ketoglutarate, a feeder of the Krebs cycle, enhances the operation of this pathway, generating ATP. When the cell has abundant ATP and GTP (the latter produced in the reaction catalyzed by succinate thiokinase), glutamate dehydrogenase is inhibited, the supply of α-ketoglutarate is reduced, and the cycle activity is depressed.

The reaction is reversible; ammonia can bind to α-ketoglutarate to form glutamate. While the direct reaction preferably uses coenzyme NAD, the reverse reaction involves the reduction of NADPH to NADP.



The utilization of different coenzymes depending on the direction of the reaction allows the independent regulation of the deamination and amination events. Due to the reversibility of the reaction, glutamate dehydrogenase acts in catabolism, as well as in the synthesis of glutamate.

Other enzymes catalyze the oxidative deamination of AA, these are flavoproteins called *amino oxidases*. Their role in human tissues is not important.

***Deamination*.** The amide groups of asparagine and glutamine are released as ammonia by hydrolysis, catalyzed by *asparaginase* and *glutaminase*, respectively, which produce aspartate and glutamate; the ammonia is protonated to give NH_4^+.

Deamination, the opposite of amination, is a type of post-translational modification (PTM) in which an amine group is removed from a protein. Enzymes that catalyze the deamination reaction are called deaminases.

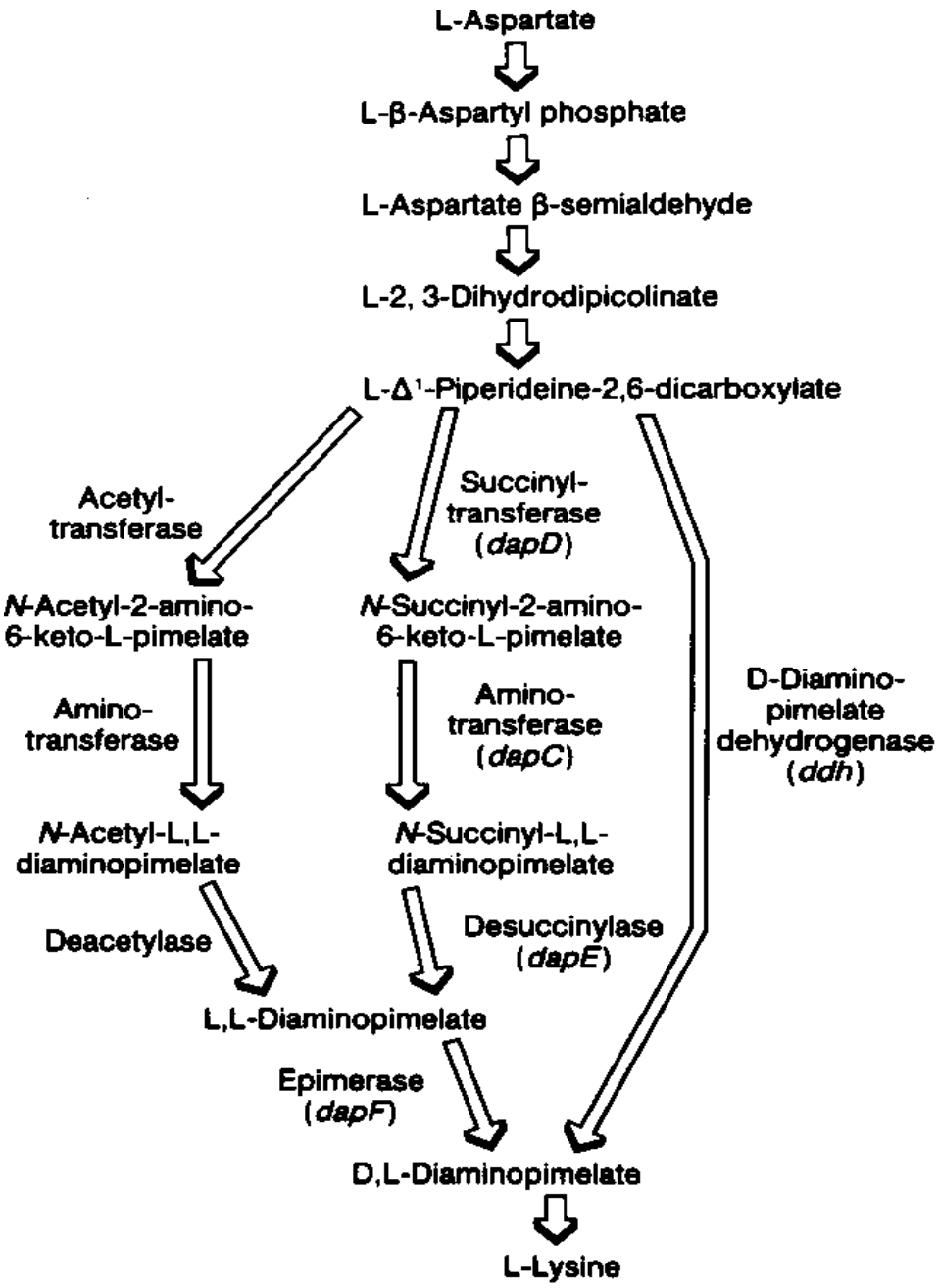
Transmethylation is a biologically important organic chemical reaction in which a methyl group is transferred from one compound to another.

An example of transmethylation is the recovery of methionine from homocysteine. In order to sustain sufficient reaction rates during metabolic stress, this reaction requires adequate levels of vitamin B_{12} and folate. Methyl tetrahydrofolate delivers methyl groups to form the active methyl form of vitamin B_{12} that is required for methylation of homocysteine. Deficiencies of vitamin B_{12} or folate cause increased levels of circulating homocysteine. Elevated homocysteine is a risk factor for cardiovascular disease and is linked to the metabolic syndrome (insulin insensitivity)

Decarboxylation reaction is defined as a chemical reaction that eliminates a carboxyl group and liberates carbon dioxide (CO_2).

Decarboxylation mostly refers to a reaction of carboxylic acids erasing a carbon atom from a chain of carbons. Carboxylation is a completely reversible process which is the first chemical step in photosynthesis, where CO_2 is added to the compound. Whereas, Enzymes that catalyse decarboxylation are known as decarboxylases.

Biosynthesis of lysine In bacteria, lysine is



synthesized via the diaminopimelate pathway. This pathway also takes part in the first and second enzymatic reactions in isoleucine, methionine and threonine biosynthesis. The first enzymatic step in the biosynthesis of these amino acids is catalyzed by aspartokinase.

Biosynthesis of glutamic acid: glutamate is synthesized from a-ketoglutaric acid, an intermediate in the citric acid cycle, by mitochondrial glutamate dehydrogenase. Glutamate is also synthesized from glutamine by glutaminase in the central nervous system. Glutamate is the most abundant excitatory neurotransmitter in the vertebrate nervous systems. Many synapses use multiple types of glutamate receptors, including ionotropic and metabotropic receptors. Three types of ionotropic glutamate receptors, AMPA, kainate, and NMDA, and three groups of metabotropic receptors are known. Glutamatergic synapses in the hippocampus, neocortex, and other parts of the brain have plasticity for long-term potentiation that enables learning and memory. Glutamate also functions as a spill-over synaptic crosstalk between synapses where released glutamate creates extrasynaptic signaling, named volume transmission. Glutamate stimulates glutamate-gated chloride channels in nematodes and arthropods. Cystine/glutamate transporters are critical regulators of ambient extracellular glutamate levels in the nervous system of Drosophila.

Glutamic acid (Glu) is synthesized from *α* KG in the TCA cycle by glutamate dehydrogenase (GDH) with NH_3 and NADPH (Figure 1.22). The glutamic acid produced inhibits GDH and also controls enzyme synthesis. Note that glutamic acid regulates the synthesis of Ppc and CS. Gutamine is synthesized from glutamate by glutamine synthetase (GS) with NH_3 and ATP (Figure 1.22). Glutamic acid may be also formed from glutamine by glutamate synthase (GOGAT) with NADPH when the NH_3 concentration is low and limiting. The ammonia assimilation or nitrogen regulation pathways are important.

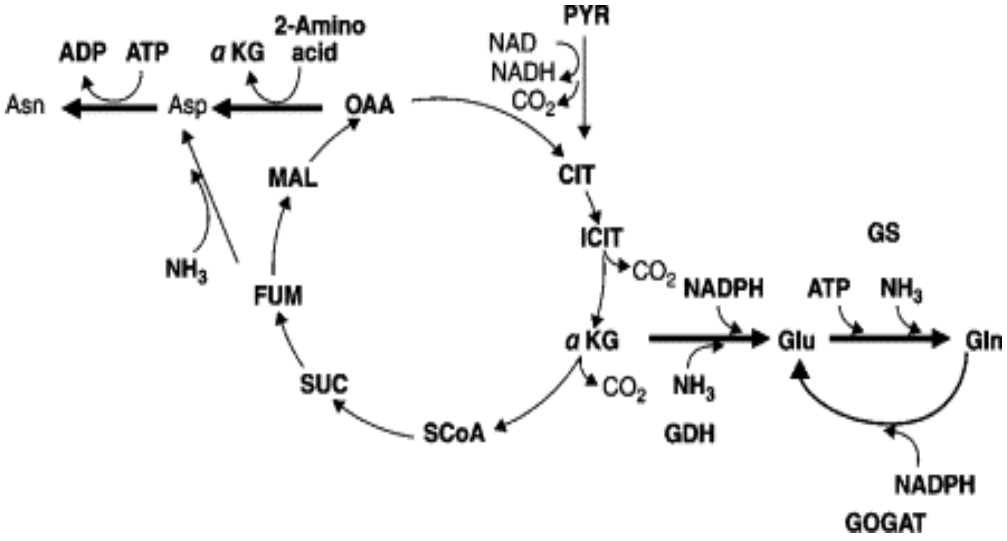


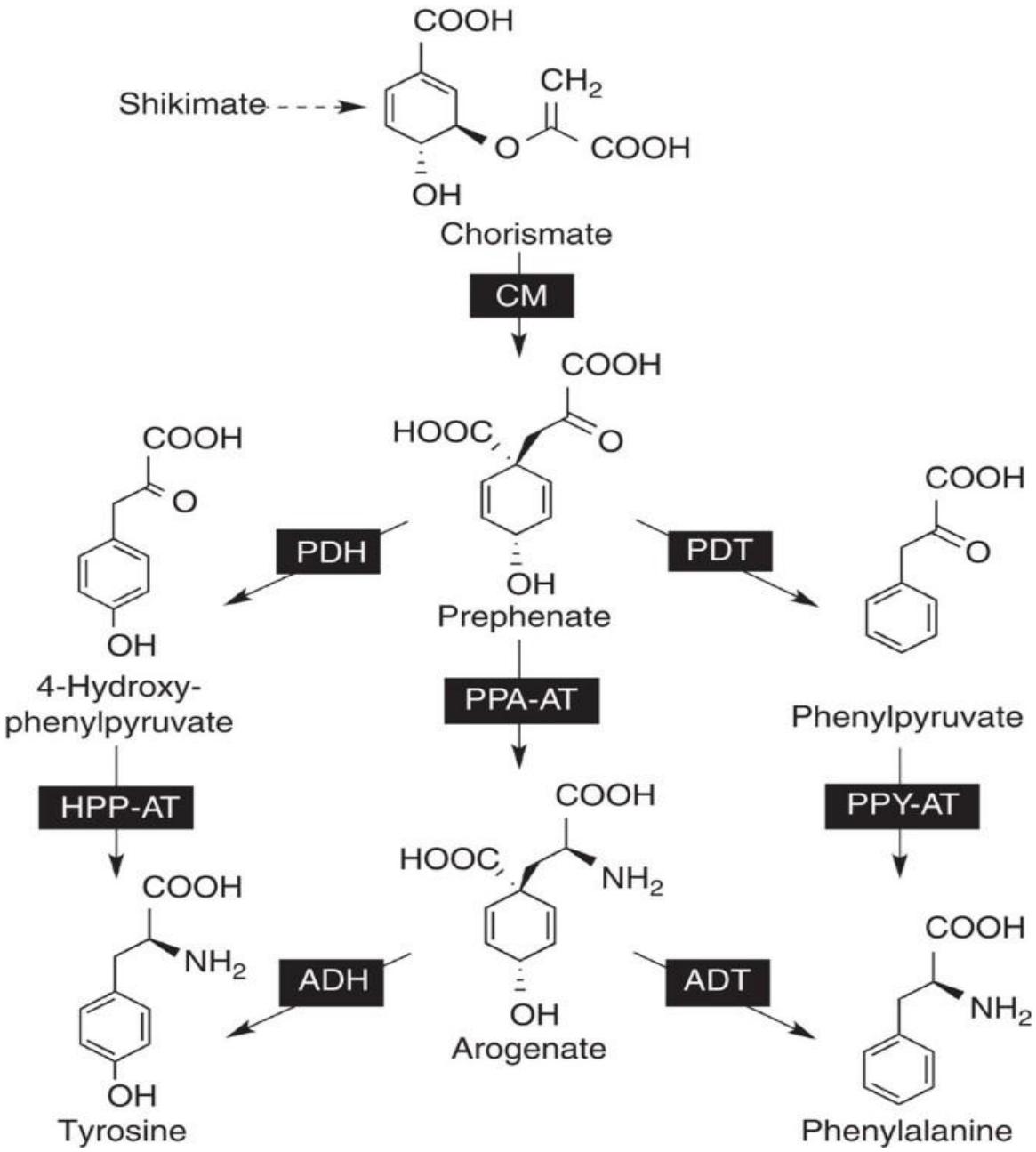
Fig: Glutamate biosynthesis

Biosynthesis of phenylalanine

Aromatic amino acids are essential constituents of proteins in all living organisms, whereas in plants, phenylalanine (Phe) and tyrosine (Tyr) also serve as precursors for thousands of vital and specialized compounds. Approximately 20–30% of photosynthetically fixed carbon is directed towards synthesis of phenylalanine, and in turn to lignin1, the central structural component of plant cell walls and the major impediment to cellulosic biofuel production. In addition to structural roles, phenylalanine-derived compounds function in plant defence and ultraviolet protection (for example, flavonoids and various phenolics), signalling (for example, isoflavonoids) and reproduction (for example, anthocyanins and phenylpropanoid/benzenoid volatiles). Moreover, tyrosine-derived metabolites, such as tocopherols (vitamin E), plastoquinone, cyanogenic glycosides and suberin have crucial roles in plant fitness.

Phenylalanine and tyrosine are synthesized from chorismate, the final product of the shikimate pathway, which is converted by chorismate mutase to prephenate. Subsequent conversion of prephenate to phenylalanine and tyrosine occurs through alternative routes. In the first (the arogenate pathway), a shared transamination reaction catalysed by prephenate aminotransferase (PPA-AT), produces L-arogenate, which can then either be dehydrated/decarboxylated to phenylalanine by arogenate dehydratase (ADT) or dehydrogenated/decarboxyled to tyrosine by arogenate dehydrogenase (ADH). In the other routes (the phenylpyruvate/4-hydroxyphenylpyruvate pathways), these reactions occur in reverse order: prephenate is first subjected to dehydration/decarboxylation or dehydrogenation/decarboxylation to form phenylpyruvate or 4-hydroxyphenylpyruvate, respectively. Then the corresponding products undergo transamination to phenylalanine and tyrosine . Most microorganisms appear to utilize only the phenylpyruvate/4-hydroxyphenylpyruvate pathways, with a few exceptions. In contrast, phenylalanine and tyrosine biosynthesis in plants has only been described to occur via the arogenate pathway. Nevertheless, overexpression of a bacterial bifunctional *chorismate mutase/prephenate dehydratase* (*PheA*) in *Arabidopsis* resulted in significantly increased phenylalanine production, implicating the presence of an aminotransferase capable of converting phenylpyruvate to phenylalanine in plants, as it occurs in the final step of the microbial phenylpyruvate pathway. Although several plant aminotransferases, mainly participating in aromatic amino-acid catabolism, have recently been described, none of them have been demonstrated to function in phenylalanine biosynthesis.

Figure 1: Proposed phenylalanine and tyrosine biosynthetic pathways in plants.



ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; CM, chorismate mutase; HPP-AT, 4-hydroxyphenylpyruvate aminotransferase; PDH, prephenate dehydrogenase; PDT, prephenate dehydratase; PPA-AT, prephenate aminotransferase; PPY-AT, phenylpyruvate aminotransferase.

Previously we demonstrated that *PhADT1* downregulation in *Petunia hybrida* flowers, which emit high levels of phenylalanine-derived volatiles, resulted in a greater decrease in phenylalanine levels compared with the effect of *PhPPA-AT* downregulation (up to 82% versus 20% reduction, respectively) Such a differential impact on phenylalanine levels from the downregulation of these individual genes was suggested to be due to either sufficient residual PPA-AT activity in the *PhPPA-AT* RNAi lines capable of sustaining the arogenate pathway, or the redirection of carbon flux from accumulated prephenate in the *PhPPA-AT* RNAi lines through the hitherto undetected alternative phenylpyruvate pathway.

Here to test these hypotheses, we generated RNAi transgenic petunia plants in which both *PhADT1* and *PhPPA-AT* genes were simultaneously downregulated. If the phenylalanine and volatile phenotypes in *PhADT1* plants can be rescued by concurrent downregulation of *PhPPA-AT*, this would be consistent with redirection of flux from prephenate through an alternative pathway. Indeed, detailed metabolic profiling of *PhADT1xPhPPA-AT* RNAi lines provides evidence in agreement with the involvement of an alternative pathway. To provide further support, we identify a petunia phenylpyruvate aminotransferase gene (designated as PhPPY-AT), downregulation of which leads to reduction of phenylalanine and phenylalanine-derived scent compounds. Extensive biochemical characterization of PhPPY-AT reveals that it preferentially converts phenylpyruvate to phenylalanine, and unexpectedly strongly favours tyrosine as the amino donor. In addition, feeding experiments with petunia petals show that the ^{15}N label from supplied ^{15}N-tyrosine is retrieved in phenylalanine, and higher phenylalanine labelling occurs in *PhADT1xPhPPA-AT* RNAi lines than in wild-type, consistent with higher flux through the phenylpyruvate pathway when the flux into the arogenate pathway is limiting. Moreover, we have shown that PhPPY-AT is a cytosolic enzyme, and when the tyrosine pool is reduced via overexpression of a cytosolic tyrosine decarboxylase, it leads to a decrease in phenylalanine levels. Taken together, these results demonstrate for the first time that the microbial-like phenylpyruvate pathway operates in plants, phenylalanine biosynthesis is not limited to plastids, and that there is an interconnection between aromatic amino-acid catabolism and biosynthesis *in planta*.

Protein biosynthesis (or **protein synthesis**) is a core biological process, occurring inside cells, balancing the loss of cellular proteins (via degradation or export) through the production of new proteins.

Proteins perform a number of critical functions as enzymes, structural proteins or hormones. Protein synthesis is a very similar process for both prokaryotes and eukaryotes but there are some distinct differences.

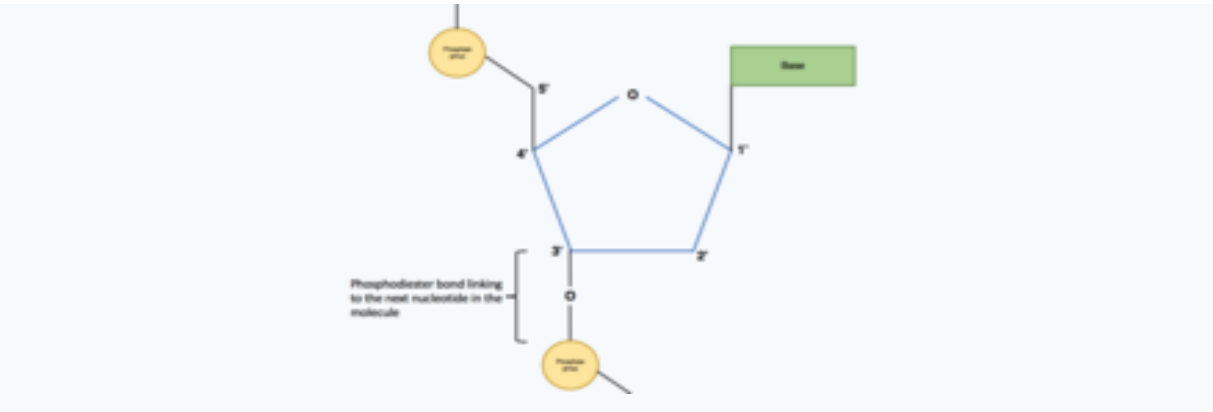
Protein synthesis can be divided broadly into two phases: transcription and translation. During transcription, a section of DNA encoding a protein, known as a gene, is converted into a template molecule called messenger RNA (mRNA). This conversion is carried out by enzymes, known as RNA polymerases, in the nucleus of the cell. In eukaryotes, this mRNA is initially produced in a premature form (pre-mRNA) which undergoes post-transcriptional modifications to produce mature mRNA. The mature mRNA is exported from the cell nucleus via nuclear pores to the cytoplasm of the cell for translation to occur. During translation, the mRNA is read by ribosomes which use the nucleotide sequence of the mRNA to determine the sequence of amino acids. The ribosomes catalyze the formation of covalent peptide bonds between the encoded amino acids to form a polypeptide chain.

Following translation the polypeptide chain must fold to form a functional protein; for example, to function as an enzyme the polypeptide chain must fold correctly to produce a functional active site. To adopt a functional three-dimensional shape, the polypeptide chain must first form a series of smaller underlying structures called secondary structures. The polypeptide chain in these secondary structures then folds to produce the overall 3D tertiary structure. Once correctly folded, the protein can undergo further maturation through different post-translational modifications, which can alter the protein's ability to function, its location within the cell (e.g. cytoplasm or nucleus) and its ability to interact with other proteins

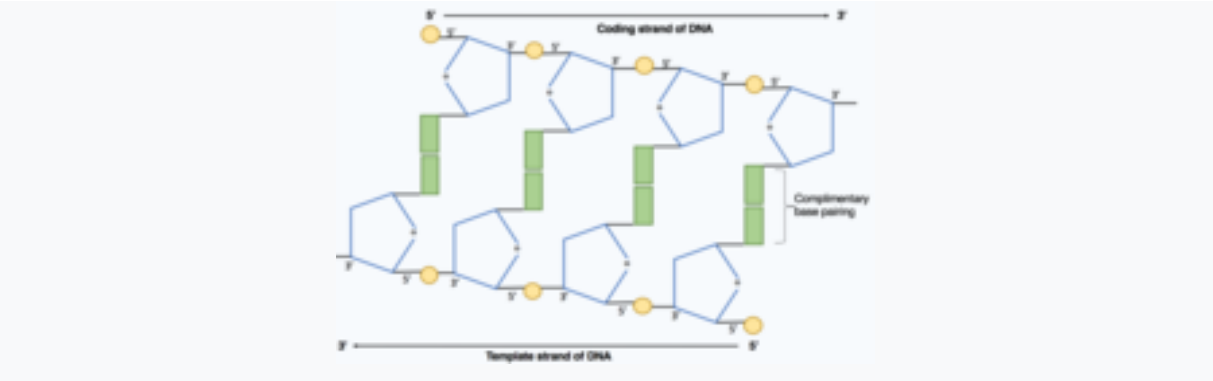
Protein biosynthesis has a key role in disease as changes and errors in this process, through underlying DNA mutations or protein misfolding, are often the underlying causes of a disease. DNA mutations change the subsequent mRNA sequence, which then alters the mRNA encoded amino acid sequence. Mutations can cause the polypeptide chain to be shorter by generating a stop sequence which causes early termination of translation. Alternatively, a mutation in the mRNA sequence changes the specific amino acid encoded at that position in the polypeptide chain. This amino acid change can impact the protein's ability to function or to fold correctly. Misfolded proteins have a tendency to form dense protein clumps, which are often implicated in diseases, particularly neurological disorders including Alzheimer's and Parkinson's disease.

Transcription

Transcription occurs in the nucleus using DNA as a template to produce mRNA. In eukaryotes, this mRNA molecule is known as pre-mRNA as it undergoes post-transcriptional modifications in the nucleus to produce a mature mRNA molecule. However, in prokaryotes post-transcriptional modifications are not required so the mature mRNA molecule is immediately produced by transcription.



Illustrates the structure of a nucleotide with the 5 carbons labelled demonstrating the 5' nature of the phosphate group and 3' nature of hydroxyl group needed to form the connective phosphodiester bonds



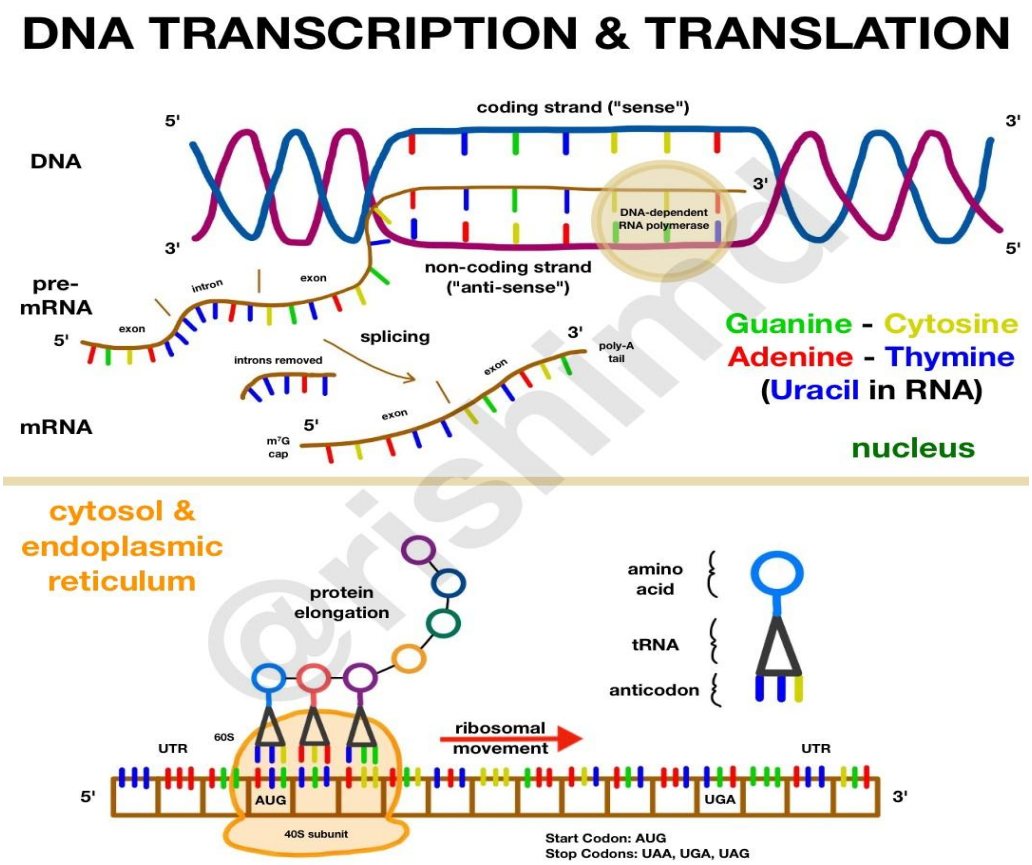
Illustrates the intrinsic directionality of DNA molecule with the coding strand running 5' to 3' and the complimentary template strand running 3' to 5'

Initially, an enzyme known as a helicase acts on the molecule of DNA. DNA has an antiparallel, double helix structure composed of two, complementary polynucleotide strands, held together by hydrogen bonds between the base pairs. The helicase disrupts the hydrogen bonds causing a region of DNA – corresponding to a gene – to unwind, separating the two DNA strands and exposing a series of bases. Despite DNA being a double-stranded molecule, only one of the strands acts as a template for pre-

mRNA synthesis; this strand is known as the template strand. The other DNA strand (which is complementary to the template strand) is known as the coding strand.

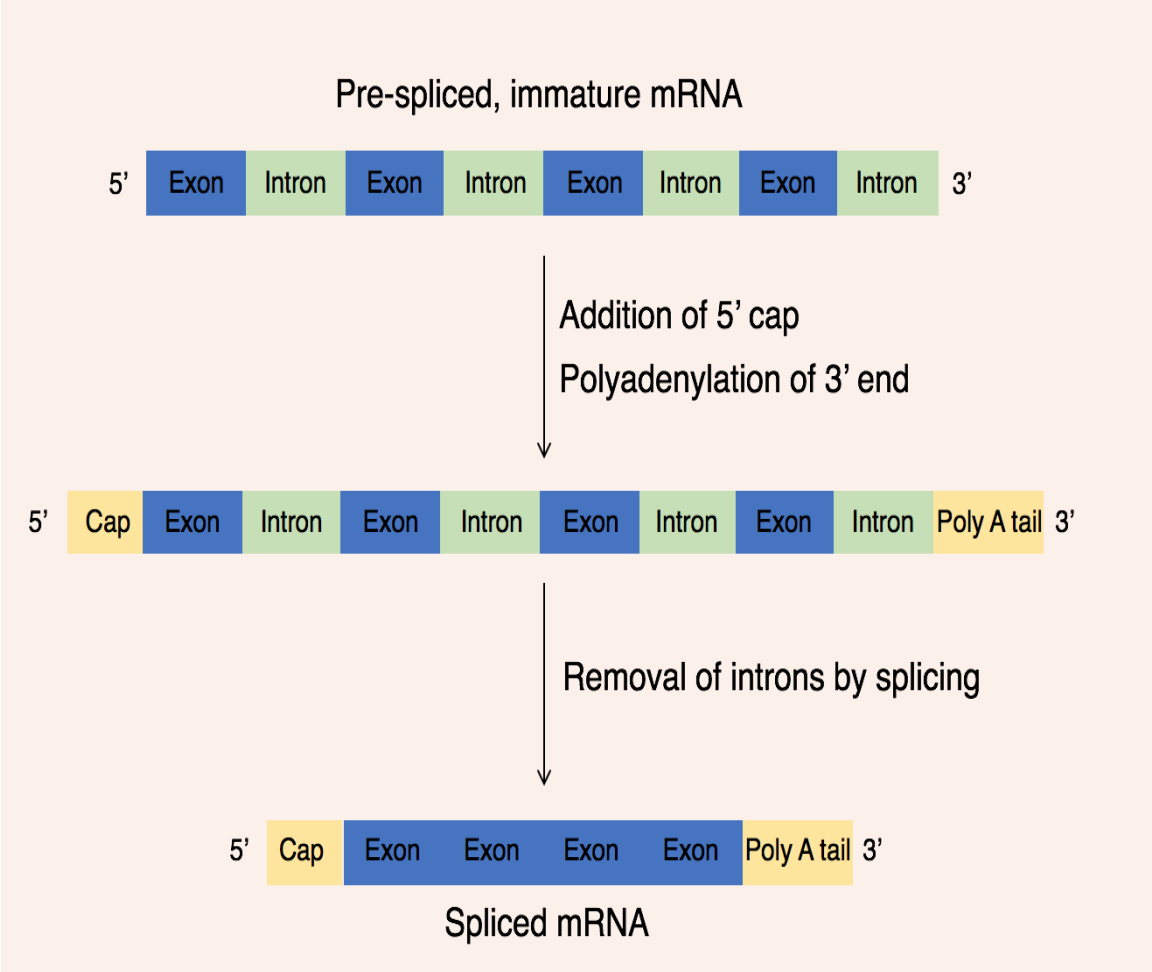
Both DNA and RNA have intrinsic directionality, meaning there are two distinct ends of the molecule. This property of directionality is due to the asymmetrical underlying nucleotide subunits, with a phosphate group on one side of the pentose sugar and a base on the other. The five carbons in the pentose sugar are numbered from 1' (where ' means prime) to 5'. Therefore, the phosphodiester bonds connecting the nucleotides are formed by joining the hydroxyl group on the 3' carbon of one nucleotide to the phosphate group on the 5' carbon of another nucleotide. Hence, the coding strand of DNA runs in a 5' to 3' direction and the complementary, template DNA strand runs in the opposite direction from 3' to 5'.

The enzyme RNA polymerase binds to the exposed template strand and reads from the gene in the 3' to 5' direction. Simultaneously, the RNA polymerase synthesizes a single strand of pre-mRNA in the 5'-to-3' direction by catalysing the formation of phosphodiester bonds between activated nucleotides (free in the nucleus) that are capable of complementary base pairing with the template strand. Behind the moving RNA polymerase the two strands of DNA rejoin, so only 12 base pairs of DNA are exposed at one time. RNA polymerase builds the pre-mRNA molecule at a rate of 20 nucleotides per second enabling the production of thousands of pre-mRNA molecules from the same gene in an hour. Despite the fast rate of synthesis, the RNA polymerase enzyme contains its own proofreading mechanism. The proofreading mechanisms allows the RNA polymerase to remove incorrect nucleotides (which are not complementary to the template strand of DNA) from the growing pre-mRNA molecule through an excision reaction. When RNA polymerasesreaches a specific DNA sequence which terminates transcription, RNA polymerase detaches and pre-mRNA synthesis is complete.



The pre-mRNA molecule synthesized is complementary to the template DNA strand and shares the same nucleotide sequence as the coding DNA strand. However, there is one crucial difference in the nucleotide composition of DNA and mRNA molecules. DNA is composed of the bases: guanine, cytosine, adenine and thymine (G, C, A and T). RNA is also composed of four bases: guanine, cytosine, adenine and uracil. In RNA molecules, the DNA base thymine is replaced by uracil which is able to base pair with adenine. Therefore, in the pre-mRNA molecule, all complementary bases which would be thymine in the coding DNA strand are replaced by uracil.

Post-transcriptional modifications:



Outlines the process of post-transcriptionally modifying pre-mRNA through capping, polyadenylation and splicing to produce a mature mRNA molecule ready for export from the nucleus.

Once transcription is complete, the pre-mRNA molecule undergoes post-transcriptional modifications to produce a mature mRNA molecule.

There are 3 key steps within post-transcriptional modifications:

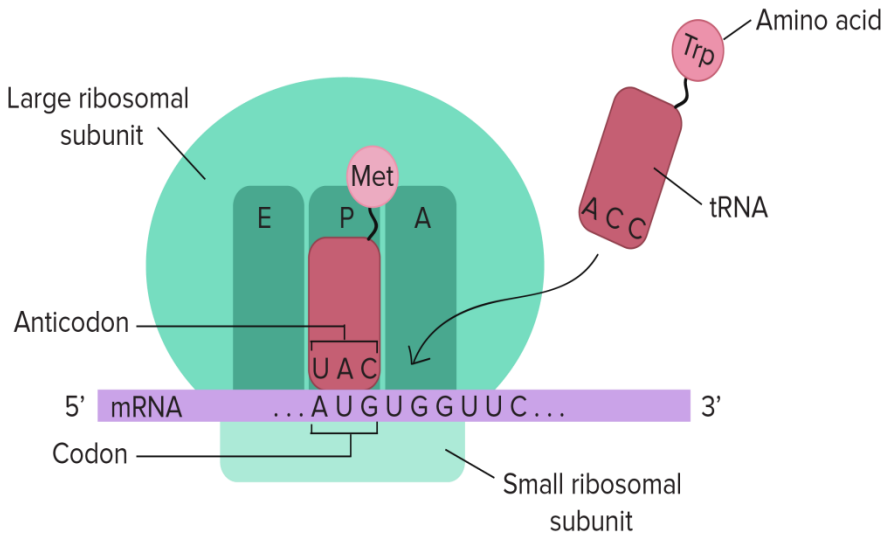
1. Addition of a 5' cap to the 5' end of the pre-mRNA molecule
2. Addition of a 3' poly(A) tail is added to the 3' end pre-mRNA molecule
3. Removal of introns via RNA splicing

The 5' cap is added to the 5' end of the pre-mRNA molecule and is composed of a guanine nucleotide modified through methylation. The purpose of the 5' cap is to prevent break down

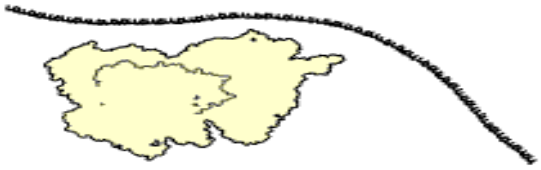
of mature mRNA molecules before translation, the cap also aids binding of the ribosome to the mRNA to start translation and enables mRNA to be differentiated from other RNAs in the cell. In contrast, the 3' Poly(A) tail is added to the 3' end of the mRNA molecule and is composed of 100-200 adenine bases. These distinct mRNA modifications enable the cell to detect that the full mRNA message is intact if both the 5' cap and 3' tail are present.

This modified pre-mRNA molecule then undergoes the process of RNA splicing. Genes are composed of a series of introns and exons, introns are nucleotide sequences which do not encode a protein while, exons are nucleotide sequences that directly encode a protein. Introns and exons are present in both the underlying DNA sequence and the pre-mRNA molecule, therefore, to produce a mature mRNA molecule encoding a protein, splicing must occur. During splicing, the intervening introns are removed from the pre-mRNA molecule by a multi-protein complex known as a spliceosome (composed of over 150 proteins and RNA). This mature mRNA molecule is then exported into the cytoplasm through nuclear pores in the envelope of the nucleus.

Translation



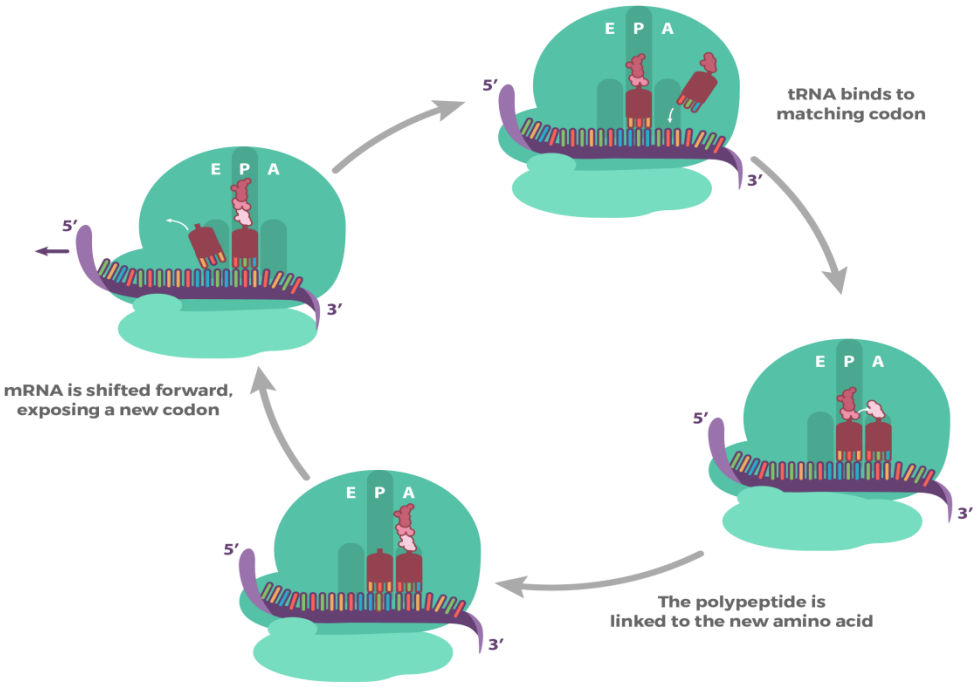
Illustrates the translation process showing the cycle of tRNA codon-anti-codon pairingand amino acid incorporation into the growing polypeptide chain by the ribosome.



A ribosome on a strand of mRNA with tRNA's arriving, performing codon-anti-codon base pairing, delivering their amino acid to the growing polypeptide chain and leaving. Demonstrates the action of the ribosome as a biological machine which functions on a nanoscale to perform translation. The ribosome moves along the mature mRNA molecule incorporating tRNA and producing a polypeptide chain.

During translation, ribosomes synthesize polypeptide chains from mRNA template molecules. In eukaryotes, translation occurs in the cytoplasm of the cell, where the ribosomes are located either free floating or attached to the endoplasmic reticulum. In prokaryotes, which lack a nucleus, the processes of both transcription and translation occur in the cytoplasm.

Ribosomes are complex molecular machines, made of a mixture of protein and ribosomal RNA, arranged into two subunits (a large and a small subunit), which surround the mRNA molecule. The ribosome reads the mRNA molecule in a 5'-3' direction and uses it as a template to determine the order of amino acids in the polypeptide chain. To translate the mRNA molecule, the ribosome uses small molecules, known as transfer RNAs (tRNA), to deliver the correct amino acids to the ribosome. Each tRNA is composed of 70-80 nucleotides and adopts a characteristic cloverleaf structure due to the formation of hydrogen bonds between the nucleotides within the molecule. There are around 60 different types of tRNAs, each tRNA binds to a specific sequence of three nucleotides (known as a codon) within the mRNA molecule and delivers a specific amino acid.

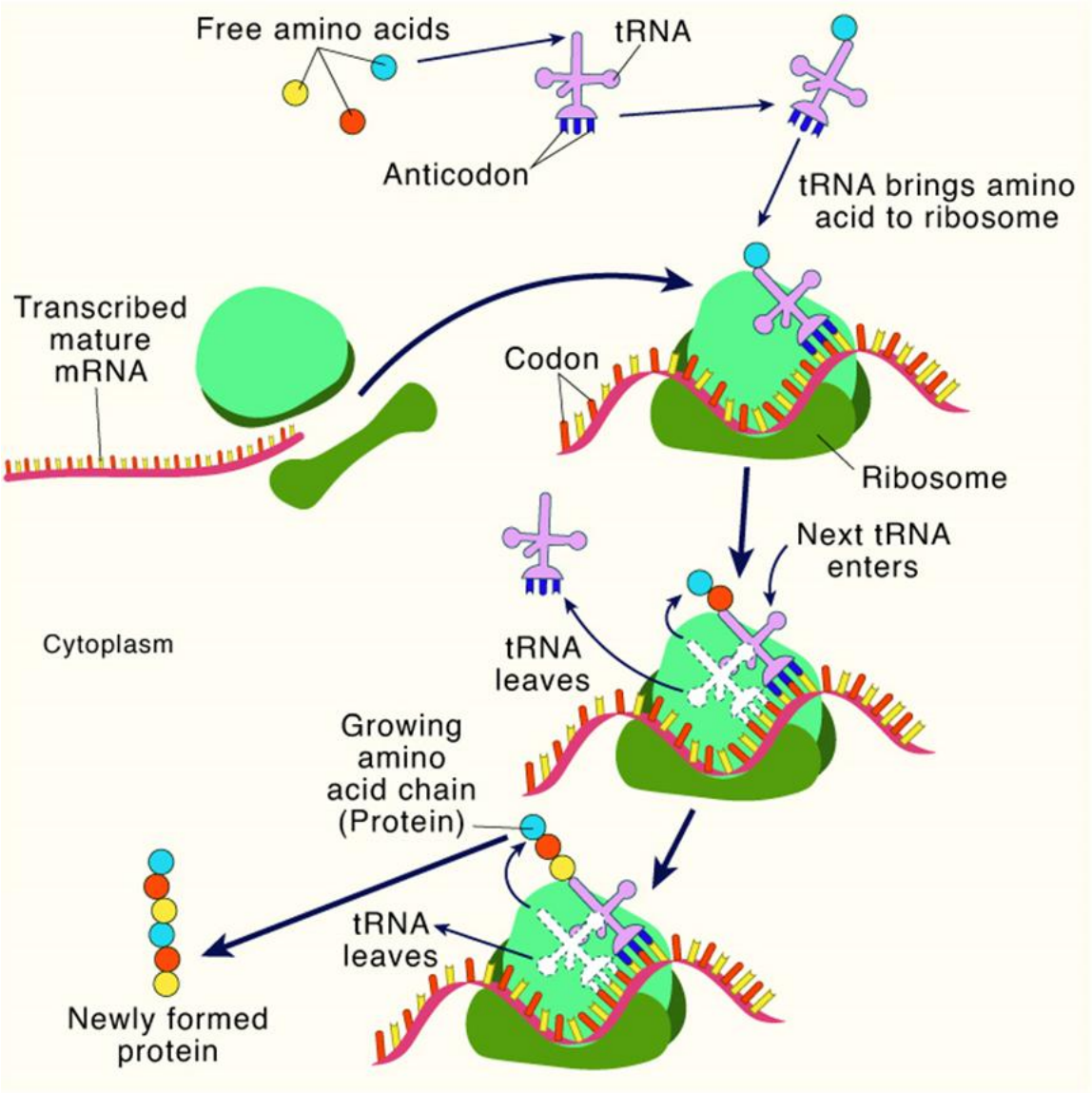


The ribosome initially attaches to the mRNA at the start codon (AUG) and begins to translate the molecule. The mRNA nucleotide sequence is read in triplets; three adjacent nucleotides in the mRNA molecule correspond to a single codon. Each tRNA has an exposed sequence of three nucleotides, known as the anticodon, which are complementary in sequence to a specific codon that may be present in mRNA. For example, the first codon encountered is the start codon composed of the nucleotides AUG. The correct tRNA with the anticodon (complementary 3 nucleotide sequence UAC) binds to the mRNA using the ribosome. This tRNA delivers the correct amino acid corresponding to the mRNA codon, in the case of the start codon, this is the amino acid methionine. The next codon (adjacent to the start codon) is then bound by the correct tRNA with complementary anticodon, delivering the next amino acid to ribosome. The ribosome then uses its peptidyl transferase enzymatic activity to catalyze the formation of the covalent peptide bond between the two adjacent amino acids.

The ribosome then moves along the mRNA molecule to the third codon. The ribosome then releases the first tRNA molecule, as only two tRNA molecules can be brought together by a single ribosome at one time. The next complementary tRNA with the correct anticodon complementary to the third codon is selected, delivering the next amino acid to the ribosome which is covalently joined to the growing polypeptide chain.

This process continues with the ribosome moving along the mRNA molecule adding up to 15 amino acids per second to the polypeptide chain. Behind the first ribosome, up to 50 additional ribosomes can bind to the mRNA molecule forming a polysome, this enables.

simultaneous synthesis of multiple identical polypeptide chains. Termination of the growing polypeptide chain occurs when the ribosome encounters a stop codon (UAA, UAG, or UGA) in the mRNA molecule. When this occurs, no tRNA can recognise it and a release factor induces the release of the complete polypeptide chain from the ribosome. Dr. Har Gobind Khorana, a scientist originating from India, decoded the RNA sequences for about 20 amino acid. He was awarded the Nobel prize in 1968, along with two other scientists, for his work.



An amino acid attaches to its respective tRNA molecule through a covalent bond during initiation. The enzyme aminoacyl-tRNA synthetase mediates this bonding between the tRNA and the amino acid, methionine, forming met-tRNA. These charged tRNAs bring the amino acids to the ribosome in proper sequence, starting with methionine.

Initiation: The Beginning

In this step, the charged Met-tRNA binds to the smaller subunit of the ribosome. The tRNA then starts reading the mRNA from the 5'-3' direction until it reaches the start codon, AUG. As soon as the tRNA finds a start codon, it binds to the P-site of the larger ribosomal subunit, forming the complete ribosome. This binding of Met-tRNA with the start codon forms the initiation complex that signals the beginning of the translation process.

In bacteria, the situation is different. There the small ribosomal subunit attaches directly to a specific sequence in the mRNA. This sequence called the Shine-Dalgarno sequence lies just before the start codon. It marks the beginning of each coding sequence, letting the ribosome find the right start codon for each gene.

Elongation: The Extension

Immediately after the met-tRNA attaches to the P-site of the ribosome, another tRNA carrying the amino acid that matches the next mRNA codon comes and sits at the A-site of the ribosome. Then, the existing amino acid binds to the amino acid of the tRNA by a peptide bond. The methionine forms the N-terminus of the polypeptide, and the other

amino acid is the C-terminus. Once bonded, the first tRNA leaves the ribosome through E-site, and the second tRNA shifts to the P-site, leaving the A-site vacant. The free tRNA again binds with another amino acid, and the elongation continues.

Termination: The End

It is the final translation step, where the polypeptide chain is released from the ribosomal complex. It occurs when either of the three stop codons UAG, UAA, or UGA in the mRNA triggers a series of events.

Stop codons are recognized by proteins called release factors, which sit perfectly into the P site. It affects the enzyme activity by adding a water molecule instead of amino acid. This activity separates the polypeptide chain from the tRNA.

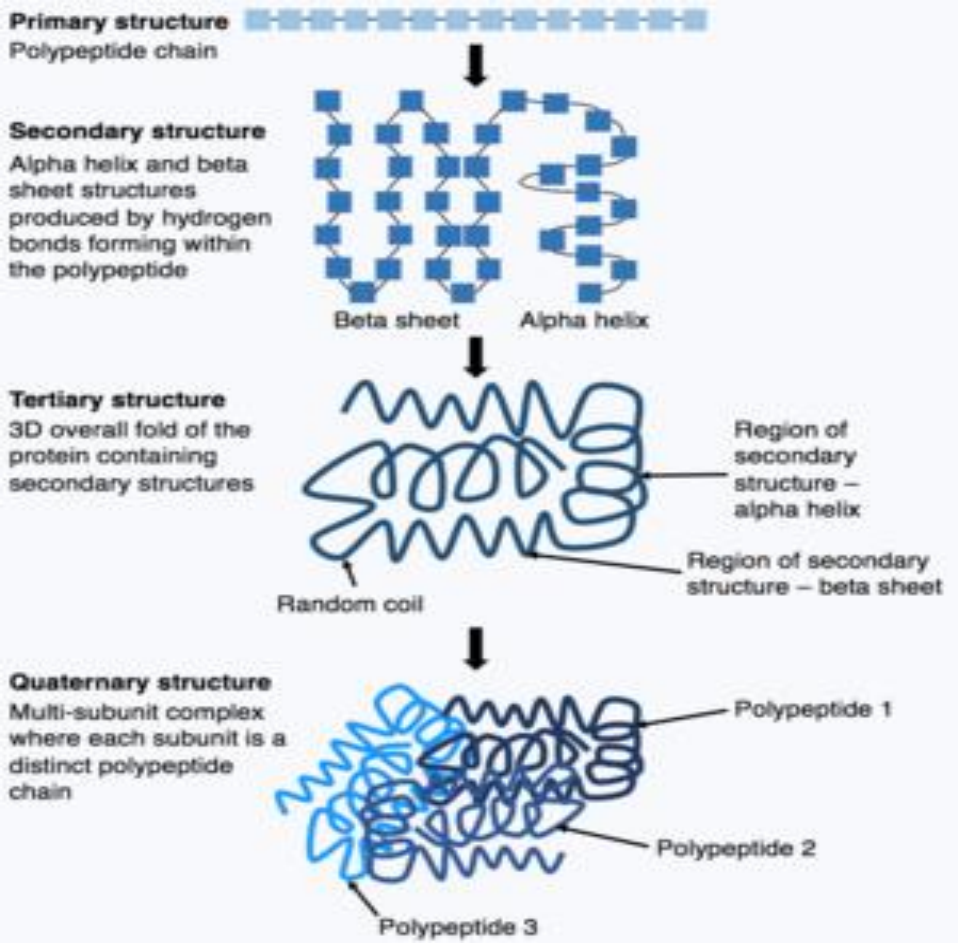
What Happens to the Ribosome after Translation

Once the translation is complete, the ribosomal complex dissociates, releasing the polypeptide chain into the cytoplasm as a newly formed protein. The subunits can again be reused during the next cycle of translation.

Protein folding

Shows the process of a polypeptide chain folding from its initial primary structure through to the quaternary structure.

Once synthesis of the polypeptide chain is complete, the polypeptide chain folds to adopt a specific structure which enables the protein to carry out its functions. The basic form of protein structure is known as the primary structure, which is simply the polypeptide chain i.e. a sequence of covalently bonded amino acids. The primary structure of a protein is encoded by a gene. Therefore, any changes to the sequence of the gene can alter the primary structure of the protein and all subsequent levels of protein structure, ultimately changing the overall structure and function.

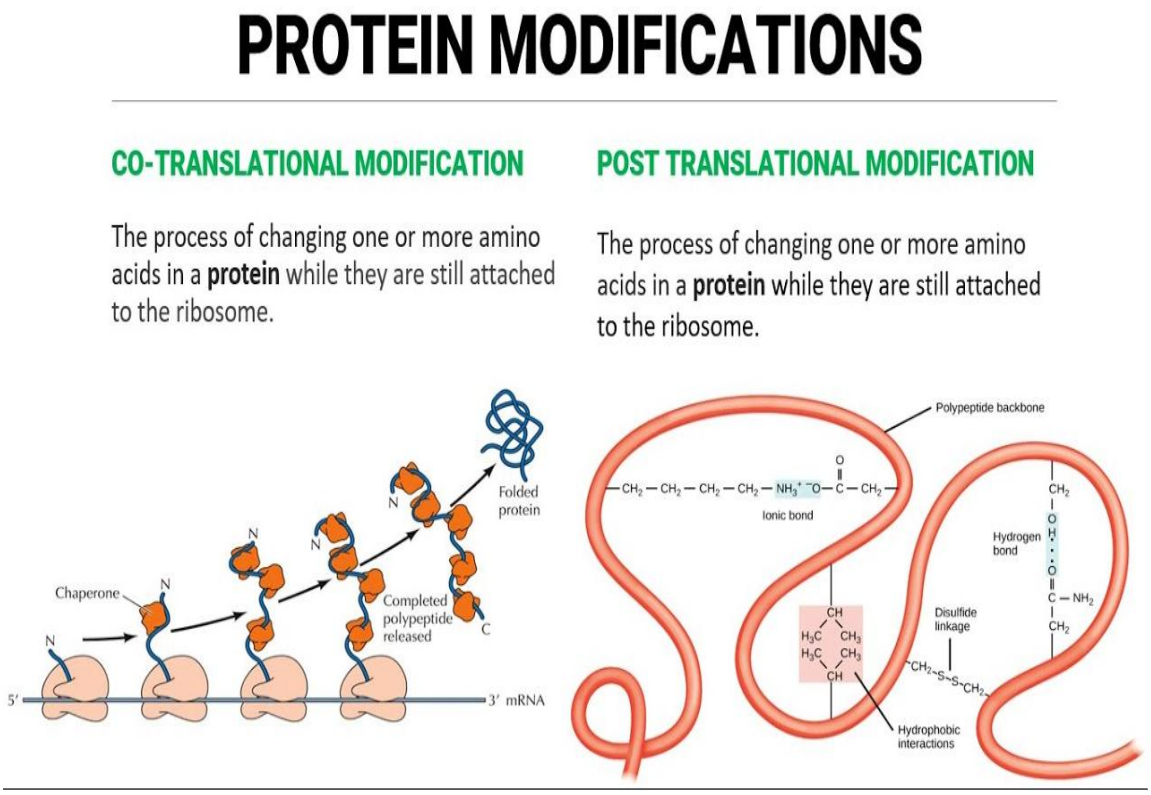


The primary structure of a protein (the polypeptide chain) can then fold or coil to form the secondary structure of the protein. The most common types of secondary structure are known as an alpha helix or beta sheet, these are small structures produced by hydrogen bonds forming within the polypeptide chain. This secondary structure then folds to produce the tertiary structure of the protein. The tertiary structure is the proteins overall 3D structure which is made of different secondary structures folding together. In the tertiary structure, key protein features e.g. the active site, are folded and formed enabling the protein to function.

Finally, some proteins may adopt a complex quaternary structure. Most proteins are made of a single polypeptide chain, however, some proteins are composed of multiple polypeptide chains (known as subunits) which fold and interact to form the quaternary structure. Hence, the overall protein is a multi-subunit complex composed of multiple folded, polypeptide chain subunits e.g. haemoglobin.

Post-translation events

There are events that follow protein biosynthesis such as **proteolysis** and protein-folding. Proteolysis refers to the cleavage of proteins by proteases and the breakdown of proteins into amino acids by the action of enzymes.



Post-translational modifications

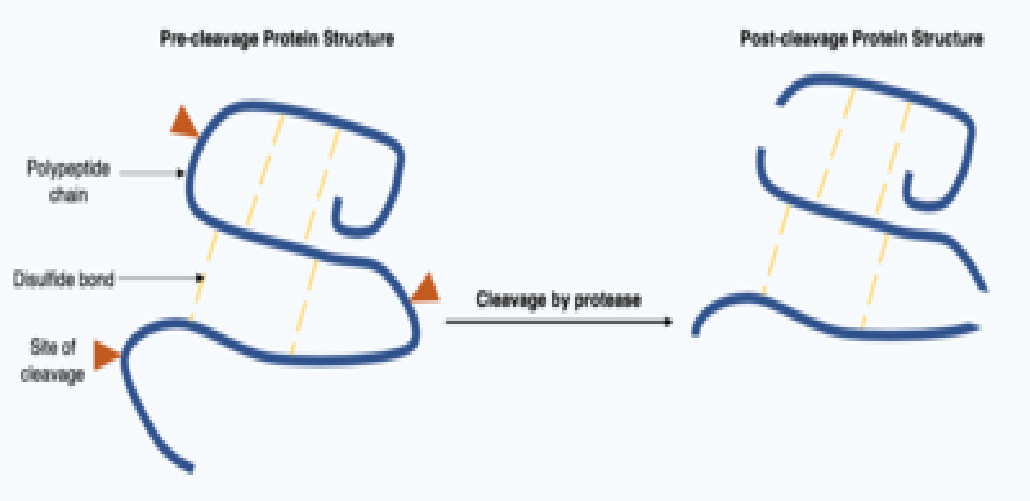
When protein folding into the mature, functional 3D state is complete, it is not necessarily the end of the protein maturation pathway. A folded protein can still undergo further processing through post-translational modifications.

There are over 200 known types of post-translational modification, these modifications can alter protein activity, the ability of the protein to interact with other proteins and where the protein is found within the cell e.g. in the cell nucleus or cytoplasm. Through post-translational modifications, the diversity of proteins encoded by the genome is expanded by 2 to 3 orders of magnitude.

There are four key classes of post-translational modification:

1. Cleavage
2. Addition of chemical groups
3. Addition of complex molecules
4. Formation of intramolecular bonds

Cleavage



Shows a post-translational modification of the protein by protease cleavage, illustrating that pre-existing bonds are retained even if when the polypeptide chain is cleaved.

Cleavage of proteins is an irreversible post-translational modification carried out by enzymes known as proteases. These proteases are often highly specific and cause hydrolysis of a limited number of peptide bonds within the target protein. The resulting shortened protein has an altered polypeptide chain with different amino acids at the start and end of the chain. This post-translational modification often alters the proteins function, the protein can be inactivated or activated by the cleavage and can display new biological activities.

Chemical groups

Phosphorylation

Reversible phosphorylation of proteins involves addition of a phosphate group on serine, threonine, or tyrosine residues and is one of the important and extensively studied PTM in both prokaryotes and eukaryotes.

Several enzymes or signaling proteins are switched ‘on’ or ‘off’ by phosphorylation or dephosphorylation. Phosphorylation is performed by enzymes called ‘kinases’, while dephosphorylation is performed by ‘phosphatases’.

Addition of a phosphate group can convert a previously uncharged pocket of protein into a negatively charged and hydrophilic protein thereby inducing conformational changes in the protein.

Phosphorylation has implications in several cellular processes, including cell cycle, growth, apoptosis and signal transduction pathways. One example is the activation of p53, a tumor suppressor protein. p53 is used in cancer therapeutics and is activated by phosphorylation of its N-terminal by several kinases.

Acetylation

Acetylation refers to addition of acetyl group in a protein. It is involved in several biological functions, including protein stability, location, synthesis; apoptosis; cancer; DNA stability. Acetylation and deacetylation of histone form a critical part of gene regulation.

Acetylation of histones reduces the positive charge on histone, reducing its interaction with the negatively charged phosphate groups of DNA, making it less tightly wound to DNA and accessible to gene transcription. Acetylation of p53, a tumor suppressor gene, is crucial for its growth suppressing properties.

Hydroxylation

This process adds a hydroxyl group (-OH) to the proteins. It is catalyzed by enzymes termed as ‘hydroxylases’ and aids in converting hydrophobic or lipophilic compounds into hydrophilic compounds.

Methylation

Methylation refers to addition of a methyl group to lysine or arginine residue of a protein. Arginine can be methylated once or twice, while lysine can be methylated once, twice, or thrice. Methylation is achieved by enzymes called methyltransferases. Methylation has been widely studied in histones wherein histone methylation can lead to gene activation or repression based on the residue that is methylated.

Complex groups

Glycosylation

Glycosylation involves addition of an oligosaccharide termed ‘glycan’ to either a nitrogen atom (N-linked glycosylation) or an oxygen atom (O-linked glycosylation). N-linked glycosylation occurs in the amide nitrogen of asparagine, while the O-linked glycosylation occurs on the oxygen atom of serine or threonine.

Carbohydrates present in the form of N-linked or O-linked oligosaccharides are present on the surface of cells and secrete proteins. They have critical roles in protein sorting, immune recognition, receptor binding, inflammation, and pathogenicity. For example, N-linked glycans on an immune cell can dictate how it migrates to specific sites. Similarly, it can also determine how a cell recognizes ‘self’ and ‘non-self’.

AMPylation

AMPylation refers to reversible addition of AMP to a protein. It involves formation of a phosphodiester bond between the hydroxyl group of the protein and the phosphate group of AMP.

Lipidation

The covalent binding of a lipid group to a protein is called lipidation. Lipidation can be further subdivided into prenylation, N-myristoylation, palmitoylation, and glycosylphosphatidylinositol (GPI)-anchor addition.

Prenylation involves the addition of isoprenoid moiety to a cysteine residue of a substrate protein. It is critical in controlling the localization and activity of several proteins that have crucial functions in biological regulation.

Myristoylation involves the addition of myristoyl group to a glycine residue by an amide bond. It has functions in membrane association and apoptosis. In palmitoylation, a palmitoyl group is added to a cysteine residue of a protein.

In GPI-anchor addition, the carboxyl-terminal signal peptide of the protein is split and replaced by a GPI anchor. Recent research in human genetics has revealed that GPI anchors are important for human health. Any defects in the assembling, attachment or remodeling of GPI anchors lead to genetic diseases known as inherited GPI deficiency.

Polypeptides

Ubiquitination

Ubiquitination involves addition of a protein found ubiquitously, termed ‘ubiquitin’, to the lysine residue of a substrate. Either a single ubiquitin molecule (monoubiquitination) or a chain of several ubiquitin molecules may be attached (polyubiquitination).

Polyubiquitinated proteins are recognized by the 26S proteasome and are subsequently targeted for proteolysis or degradation. Monoubiquitinated proteins may influence cell tracking and endocytosis.

Protein cleavage

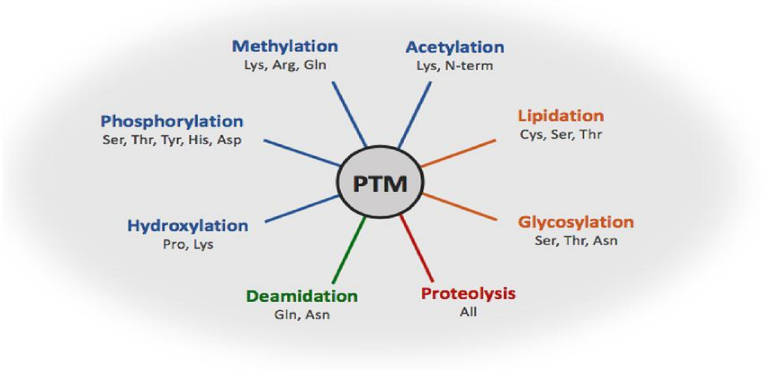
Proteolysis

Proteolysis refers to breakdown of proteins into smaller polypeptides or amino acids. For example, removal of N-terminal methionine, a signal peptide, after translation leads to conversion of an inactive or non-functional protein to an active one.

Amino acid modification

Deamidation

Deamidation is the removal or conversion of asparagine or glutamine residue to another functional group. Asparagine is converted to aspartic acid or isoaspartic acid, while glutamine is converted to glutamic acid or pyroglutamic acid. This modification can change the protein structure, stability, and function.



An overview of the most common post-translational modifications in bacteria, showing the amino acid side chains which are most frequently modified.

Lipid metabolism:

Lipids are universal biological molecules. Not only does this broad class of compounds represent the primary structural component of biological membranes in all organisms, they also serve a number of vital roles in microorganisms. Among these, lipids can be metabolized by microbes for use as a primary energy source. Although not stated explicitly, the “Organic Acid Metabolism” atom in this module introduces the concept of lipid metabolism by describing the process of fatty acid metabolism through β-oxidation.

This atom will expand on the metabolic pathway that enables degradation and utilization of lipids. Fatty acids are the building blocks of lipids. They are made of a hydrocarbon chain of variable length that terminates with a carboxylic acid group (-COOH). The fatty acid structure (see below) is one of the most fundamental categories of biological lipids. It is commonly used as a building block of more structurally complex lipids (such as phospholipids and triglycerides). When metabolized, fatty acids yield large quantities of ATP, which is why these molecules are important energy sources. Lipids are an energy and carbon source. Before complex lipids can be used to produce energy, they must first be hydrolyzed. This requires the activity of hydrolytic enzymes called lipases, which release fatty acids from derivatives such as phospholipids. These fatty acids can then enter a dedicated pathway that promotes step-wise lipid processing that ultimately yields acetyl-CoA, a critical metabolite that conveys carbon atoms to the TCA cycle to be oxidized for energy production

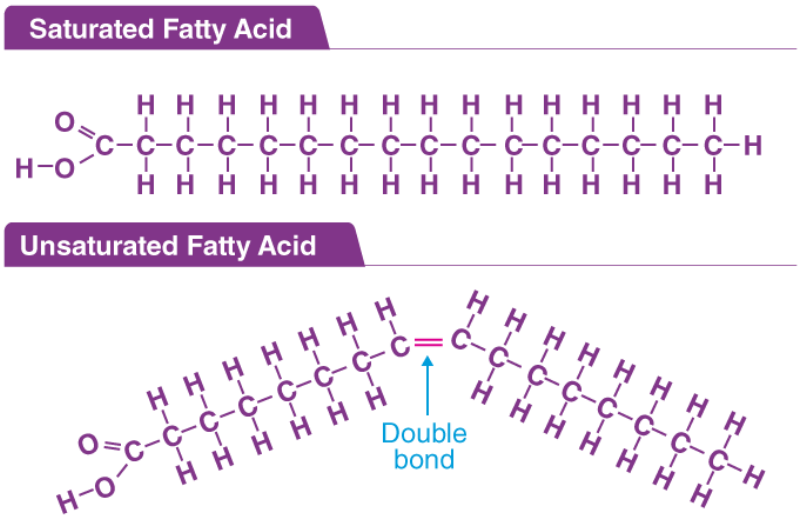


Figure: **An example of a fatty acid**: A fatty acid is a carboxylic acid with a long aliphatic tail that may be either saturated or unsaturated. The molecule shown here is the eight-carbon saturated fatty acid known as octanoic acid (or caprylic acid).

β-oxidation

The metabolic process by which fatty acids and their lipidic derivatives are broken down is called β-oxidation. This process bears significant similarity to the mechanism by which fatty acids are synthesized, except in reverse.

In brief, the oxidation of lipids proceeds as follows: two-carbon fragments are removed sequentially from the carboxyl end of the fatty acid after dehydrogenation, hydration, and oxidation to form a keto acid, which is then cleaved by thiolysis. The acetyl-CoA molecule liberated by this process is eventually converted into ATP through the TCA cycle..

β-oxidation can be broken down into a series of discrete steps:

1. Activation: Before fatty acids can be metabolized, they must be "activated. " This activation step involves the addition of a coenzyme A (CoA) molecule to the end of a long-chain fatty acid, after which the activated fatty acid (fatty acyl -CoA) enters the β-oxidation pathway.
2. Oxidation: The initial step of β-oxidation is catalyzed by acyl-CoA dehydrogenase, which oxidizes the fatty acyl-CoA molecule to yield enoyl-CoA. As a result of this process, a trans double bond is introduced into the acyl chain.
3. Hydration: In the second step, enoyl-CoA hydratase hydrates the double bond introduced in the previous step, yielding an alcohol (-C-OH).
4. Oxidation: Hydroxyacyl-CoA dehydrogenase oxidizes the alcohol formed in the previous step to a carbonyl (-C=O).
5. Cleavage: A thiolase then cleaves off acetyl-CoA from the oxidized molecule, which also yields an acyl-CoA that is two carbons shorter than the original molecule that entered the β-oxidation pathway.

This cycle repeats until the fatty acid has been completely reduced to acetyl-CoA, which is fed through the TCA cycle to ultimately energy in the yield cellular form of ATP.

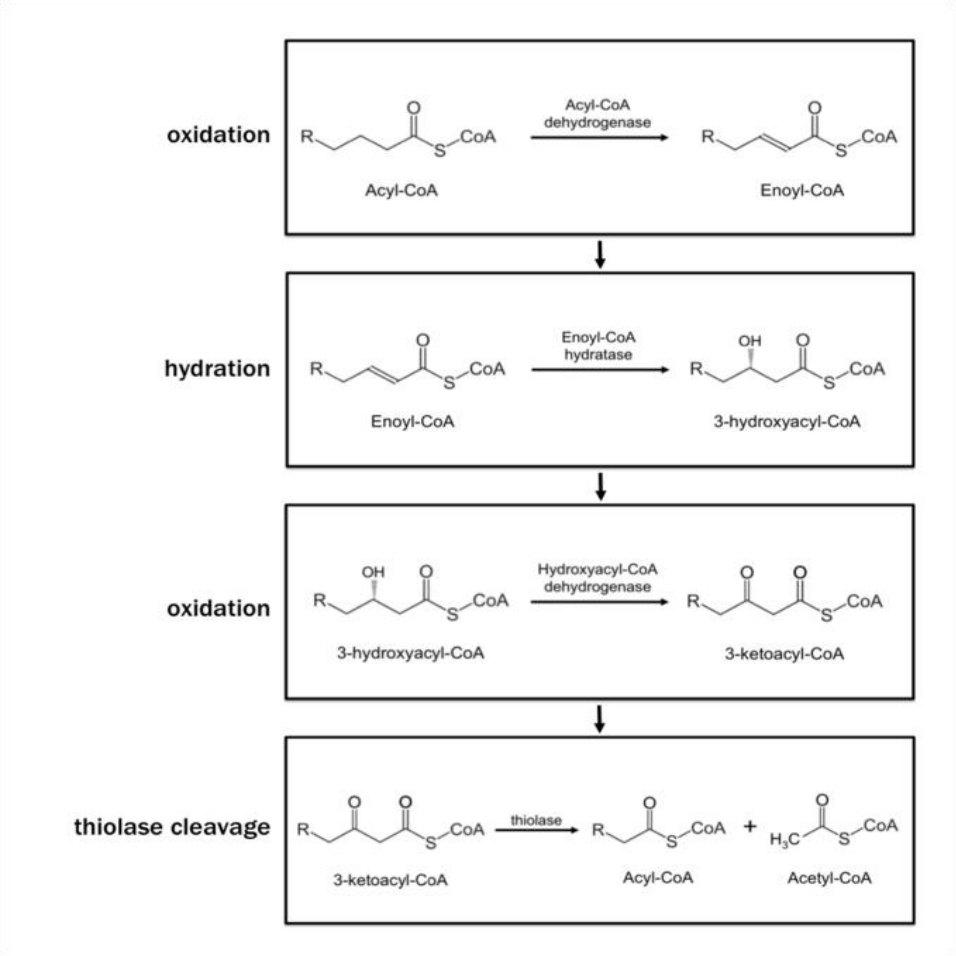


Figure: **β-oxidation**: The sequential steps of the β-oxidation pathway.

In the liver oxaloacetate can be wholly or partially diverted into the gluconeogenic pathway during fasting, starvation, a low carbohydrate diet, prolonged strenuous exercise, and in uncontrolled type 1 diabetes mellitus. Under these circumstances, oxaloacetate is hydrogenated to malate, which is then removed from the mitochondria of the liver cells to be converted into glucose in the cytoplasm of the liver cells, from where it is released into the blood.[10] In the liver, therefore, oxaloacetate is unavailable for condensation with acetyl-CoA when significant gluconeogenesis has been stimulated by low (or absent) insulin and high glucagon concentrations in the blood. Under these conditions, acetyl-CoA is diverted to the formation of acetoacetate and beta-hydroxybutyrate.[10] Acetoacetate, beta-hydroxybutyrate, and their spontaneous breakdown product, acetone, are frequently, but confusingly, known as ketone bodies (as they are not "bodies" at all, but water-soluble chemical substances). The ketones are released by the liver into the blood. All cells with

mitochondria can take up ketones from the blood and reconvert them into acetyl-CoA, which can then be used as fuel in their citric acid cycles, as no other tissue can divert its oxaloacetate into the gluconeogenic pathway in the way that this can occur in the liver. Unlike free fatty acids, ketones can cross the blood–brain barrier and are therefore available as fuel for the cells of the central nervous system, acting as a substitute for glucose, on which these cells normally survive.[10] The occurrence of high levels of ketones in the blood during starvation, a low carbohydrate diet, prolonged heavy exercise, or uncontrolled type 1 diabetes mellitus is known as ketosis, and, in its extreme form, in out-of-control type 1 diabetes mellitus, as ketoacidosis.

The glycerol released by lipase action is phosphorylated by glycerol kinase in the liver (the only tissue in which this reaction can occur), and the resulting glycerol 3-phosphate is oxidized to dihydroxyacetone phosphate. The glycolytic enzyme triose phosphate isomerase converts this compound to glyceraldehyde 3-phosphate, which is oxidized via glycolysis, or converted to glucose via gluconeogenesis.

Fatty acids as an energy source

Fatty acids, stored as triglycerides in an organism, are a concentrated source of energy because they contain little oxygen and are anhydrous. The energy yield from a gram of fatty acids is approximately 9 kcal (37 kJ), much higher than the 4 kcal (17 kJ) for carbohydrates. Since the hydrocarbon portion of fatty acids is hydrophobic, these molecules can be stored in a relatively anhydrous (water-free) environment. Carbohydrates, on the other hand, are more highly hydrated. For example, 1 g of glycogen binds approximately 2 g of water, which translates to 1.33 kcal/g (4 kcal/3 g). This means that fatty acids can hold more than six times the amount of energy per unit of stored mass. Put another way, if the human body relied on carbohydrates to store energy, then a person would need to carry 31 kg (67.5 lb) of hydrated glycogen to have the energy equivalent to 4.6 kg (10 lb) of fat.

Hibernating animals provide a good example for utilization of fat reserves as fuel. For example, bears hibernate for about 7 months, and during this entire period, the energy is derived from degradation of fat stores. Migrating birds similarly build up large fat reserves before embarking on their intercontinental journeys.

The fat stores of young adult humans average between about 10–20 kg, but vary greatly depending on gender and individual disposition. By contrast, the human body stores only about 400 g of glycogen, of which 300 g is locked inside the skeletal muscles and is unavailable to the body as a whole.

The 100 g or so of glycogen stored in the liver is depleted within one day of starvation. Thereafter the glucose that is released into the blood by the liver for general use by the body tissues has to be synthesized from the glucogenic amino acids and a few other gluconeogenic substrates, which do not include fatty acids. Nonetheless, lipolysis releases glycerol which can enter the pathway of gluconeogenesis.

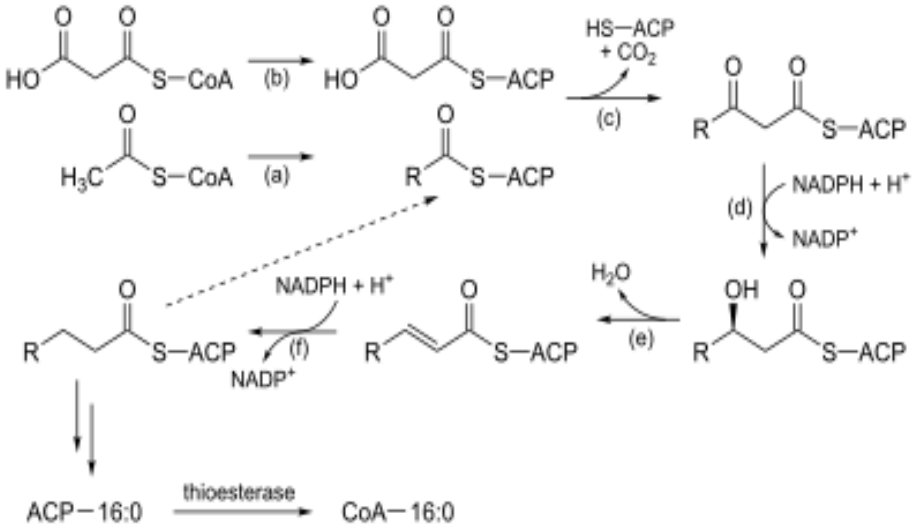
Fatty acid synthesis:

Much like beta-oxidation, straight-chain fatty acid synthesis occurs via the six recurring reactions shown below, until the 16-carbon palmitic acid is produced.

The diagrams presented show how fatty acids are synthesized in microorganisms and list the enzymes found in Escherichia coli. These reactions are performed by fatty acid synthase II (FASII), which in general contains multiple enzymes that act as one complex. FASII is present in prokaryotes, plants, fungi, and parasites, as well as in mitochondria.

In animals as well as some fungi such as yeast, these same reactions occur on fatty acid synthase I (FASI), a large dimeric protein that has all of the enzymatic activities required to create a fatty acid. FASI is less efficient than FASII; however, it allows for the formation of more molecules, including "medium-chain" fatty acids via early chain termination. Enzymes, acyltransferases and transacylases, incorporate fatty acids in phospholipids, triacylglycerols, etc. by transferring fatty acids between an acyl acceptor and donor. They also have the task of synthesizing bioactive lipids as well as their precursor molecules.

Once a 16:0 carbon fatty acid has been formed, it can undergo a number of modifications, resulting in desaturation and/or elongation. Elongation, starting with stearate (18:0), is performed mainly in the endoplasmic reticulum by several membrane-bound enzymes. The enzymatic steps involved in the elongation process are principally the same as those carried out by fatty acid synthesis, but the four principal successive steps of the elongation are performed by individual proteins, which may be physically associated.



Synthesis of saturated fatty acids via Fatty Acid Synthase II in E. coli

Note that during fatty synthesis the reducing agent is NADPH, whereas NAD is the oxidizing agent in beta-oxidation (the breakdown of fatty acids to acetyl-CoA). This difference exemplifies a general principle that NADPH is consumed during biosynthetic reactions, whereas NADH is generated in energy-yielding reactions.[34] (Thus NADPH is also required for the synthesis of cholesterol from acetyl-CoA; while NADH is generated during glycolysis.) The source of the NADPH is two-fold. When malate is oxidatively decarboxylated by "NADP+-linked malic enzyme" pyruvate, CO2 and NADPH are formed. NADPH is also formed by the pentose phosphate pathway which converts glucose into ribose, which can be used in synthesis of nucleotides and nucleic acids, or it can be catabolized to pyruvate.

Glycolytic end products are used in the conversion of carbohydrates into fatty acids

Main article: Citric acid cycle Glycolytic end products are used in the conversion of carbohydrates into fatty acids

In humans, fatty acids are formed from carbohydrates predominantly in the liver and adipose tissue, as well as in the mammary glands during lactation. The pyruvate produced by glycolysis is an important intermediary in the conversion of carbohydrates into fatty acids and cholesterol. This occurs via the conversion of pyruvate into acetyl-CoA in the mitochondrion. However, this acetyl CoA needs to be transported into cytosol where the synthesis of fatty acids and cholesterol occurs. This cannot occur directly.

To obtain cytosolic acetyl-CoA, citrate (produced by the condensation of acetyl CoA with oxaloacetate) is removed from the citric acid cycle and carried across the inner mitochondrial membrane into the cytosol. There it is cleaved by ATP citrate lyase into acetyl-CoA and oxaloacetate. The oxaloacetate is returned to mitochondrion as malate (and then converted back into oxaloacetate to transfer more acetyl-CoA out of the mitochondrion). The cytosolic acetyl-CoA is carboxylated by acetyl CoA carboxylase into malonyl CoA, the first committed step in the synthesis of fatty acids.

Regulation of fatty acid synthesis

Acetyl-CoA is formed into malonyl-CoA by acetyl-CoA carboxylase, at which point malonyl-CoA is destined to feed into the fatty acid synthesis pathway. Acetyl-CoA carboxylase is the point of regulation in saturated straight-chain fatty acid synthesis, and is subject to both phosphorylation and allosteric regulation. Regulation by phosphorylation occurs mostly in mammals, while allosteric regulation occurs in most organisms. Allosteric control occurs as feedback inhibition by palmitoyl-CoA and activation by citrate. When there are high levels of palmitoyl-CoA, the final product of saturated fatty acid synthesis, it allosterically inactivates acetyl-CoA carboxylase to prevent a build-up of fatty acids in cells. Citrate acts to activate acetyl-CoA carboxylase under high levels, because high levels indicate that there is enough acetyl-CoA to feed into the Krebs cycle and produce energy.

High plasma levels of insulin in the blood plasma (e.g. after meals) cause the dephosphorylation and activation of acetyl-CoA carboxylase, thus promoting the formation of malonyl-CoA from acetyl-CoA, and consequently the conversion of carbohydrates into fatty acids, while epinephrine and glucagon (released into the blood during starvation and exercise) cause the phosphorylation of this enzyme, inhibiting lipogenesis in favor of fatty acid oxidation via beta-oxidation.

Disorders

Disorders of fatty acid metabolism can be described in terms of, for example, hypertriglyceridemia (too high level of triglycerides), or other types of hyperlipidemia. These may be familial or acquired.

Familial types of disorders of fatty acid metabolism are generally classified as inborn errors of lipid metabolism. These disorders may be described as fatty acid oxidation disorders or as a lipid storage disorders, and are any one of several inborn errors of metabolism that result from enzyme or transport protein defects affecting the ability of the body to oxidize fatty acids in order to produce energy within muscles, liver, and other cell types. When a fatty acid oxidation disorder affects the muscles, it is a metabolic myopathy.

Moreover, cancer cells can display irregular fatty acid metabolism with regard to both fatty acid synthesis and mitochondrial fatty acid oxidation (FAO) that are involved in diverse aspects of tumorigenesis and cell growth.

Nucleic acid metabolism:

They inhibit nucleic acid synthesis by inhibiting thymidylate synthase. Thymidylate synthase methylates deoxyuracilmonophoshate (dUMP) into deoxythyminemonophosphate (dTMP). Fluoropyrimidines, such as fluorocytosine (5-FC) and 5-fluorouracil (5-FU), are synthetic structural analogs of the DNA nucleotide cytosine. 5-FC itself has no antifungal activity, and its fungistatic properties are dependent upon the conversion into 5-FU. The drug rapidly enters the fungal cell through specific transporters, such as cytosine permeases or pyrimidine transporters, before it is converted into 5-FU by the cytosine deaminase .These are also included in the category of *Antimetabolites*.

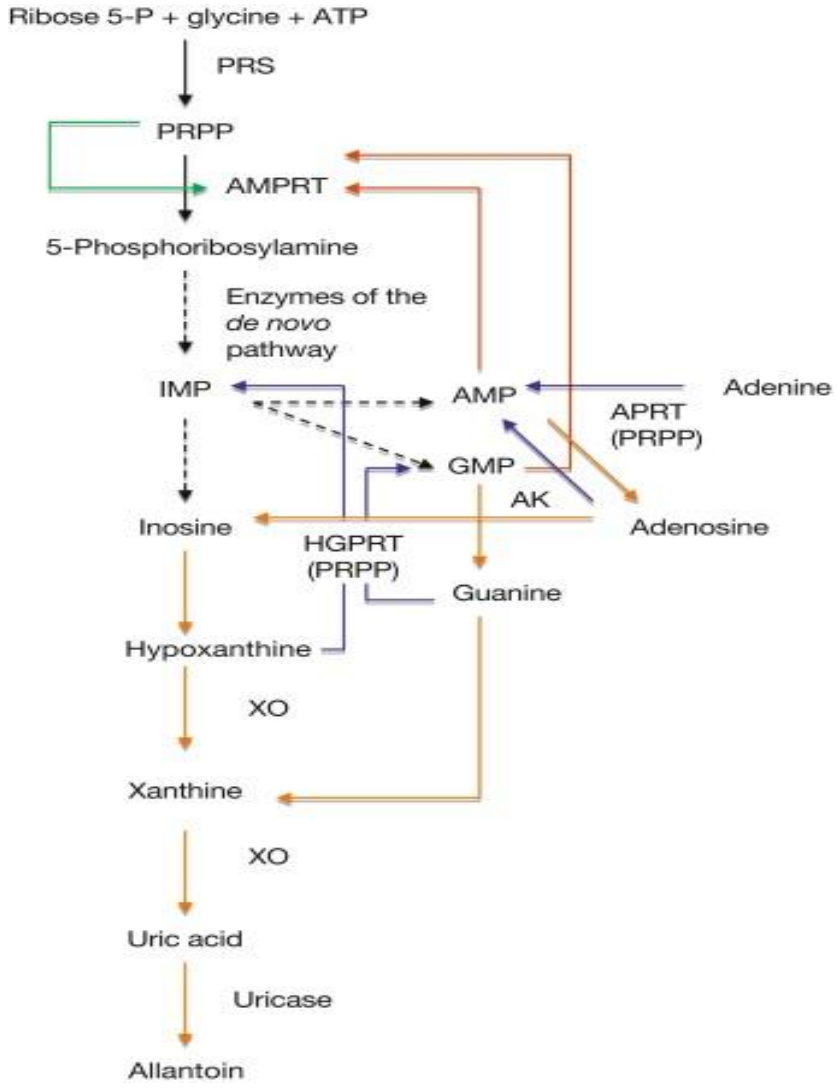
Purine Metabolism

Purines are central molecules for nucleic acid synthesis, energy production, and biological signaling. The end point of purine metabolism is the production of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) through the intermediate synthesis of four ribonucleotides: adenosine monophosphate, inosine monophosphate, xanthosine monophosphate, and guanosine monophosphate (AMP, IMP, XMP, and GMP, respectively).

The three processes which generate the body purine pool are: the '*de novo*' purine synthesis from smaller organic molecules, salvage of preformed purine bases, and purine uptake from the extracellular medium (dietary) using the salvage metabolic pathways.

The *de novo* purine synthesis is a tightly regulated multistep anabolic process which occurs mainly in the liver, although most cells are capable of performing it. Its first step is the synthesis of the molecule phosphoribosyl pyrophosphate (PRPP) from ribose-5-phosphate, glycine, and adenosine triphosphate (ATP). This reaction is catalyzed by the enzyme phosphoribosylpyrophosphate synthetase. PRPP offers the purine ring molecular backbone on which several small precursors are incorporated in a sequence of ten reactions leading to the synthesis of inosinic acid (IMP). Control of purine synthesis through the *de novo* pathway is mainly exerted through the allosteric domain of the enzyme amidophosphoribosyltransferase (AMPRT), which converts PRPP in the next intermediary product on its way into IMP, 5-phosphoribosylamin. AMPRT is stimulated by increased PRPP substrate concentrations and is subject to feedback inhibition by the purine nucleotides .

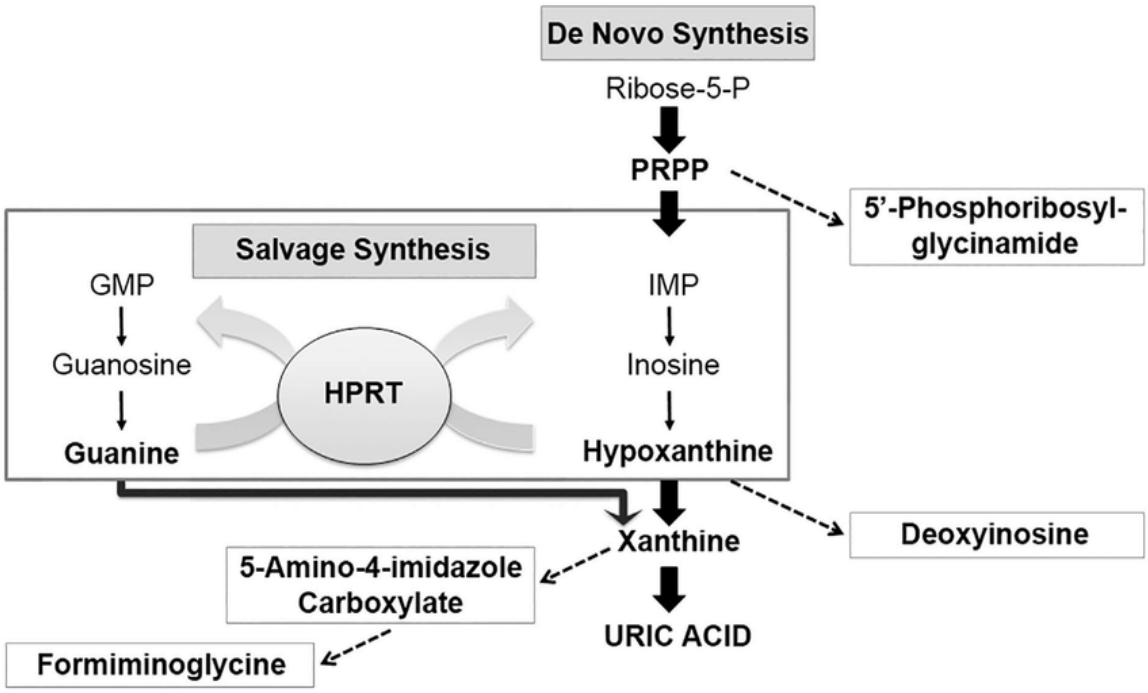
Tight regulation of the *de novo* synthesis is necessary because this is a metabolically demanding pathway which requires, for the generation of each IMP molecule, the expenditure of four amino acids, two folates, one PRPP molecule, and three ATP molecules.



Human purine recycling or salvage of preformed purine bases is mediated by three different enzymes: hypoxanthine-guanine phosphoribosyltransferase (HGPRT), adenine-phosphoribosyltransferase (APRT), and adenosine kinase (AK) .It is estimated that 90% of the free purines generated in the intracellular metabolism are recycled rather than degraded or excreted, ensuring the efficient reuse of preformed purines and energy conservation. HGPRT recycles hypoxanthine and guanine into IMP and GMP, respectively. APRT, structurally and functionally similar to HGPRT, recycles free adenine into AMP. HGPRT and APRT need PRPP as a cosubstrate for purine base recycling. AK phosphorylates adenosine to generate AMP. AK prevents the diffusion of free adenosine from the cell or its entrance into the urate catabolic pathway, maintaining the adenosine pool.

The salvage pathway metabolic mechanisms are also essential for the utilization of purines absorbed after dietary digestion. Free purines are generated from DNA and RNA degradation after digestion of purine-rich vegetables and animal products. These bases are absorbed from the gut and incorporated into salvage pathways and the cellular purine pool in the liver through the action of HGPRT and APRT. Salvage enzyme deficiencies result in an inability to recycle intracellular purines and incorporate purines from extracellular sources.

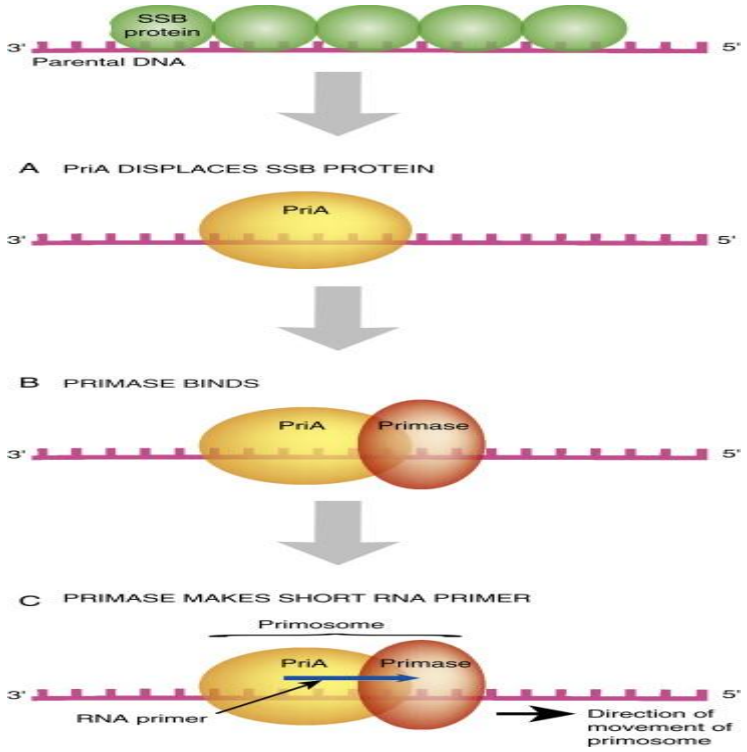
Purine nucleotide degradation starts with nucleoside formation (adenosine, inosine, and guanosine) through removal of phosphate moieties mainly catalyzed by the 5′-nucleotidase enzymes. Inosine and guanosine, through the action of purine-nucleoside phosphorylase, are transformed into the purine bases hypoxanthine and guanine, respectively. Adenosine is converted into inosine by the enzyme adenosine deaminase. Guanine, on the other hand, is converted to xanthine by guanine deaminase. Finally, the purine bases hypoxanthine and xanthine (oxypurines) are oxidized to urate by the enzyme xanthine oxidoreductase, one of its isoforms being xanthine oxidase, which is a substrate for commonly used urate-lowering gout medications. In general, the activity of these enzymes is regulated by substrate availability. In humans and primates, urate is the final product of purine metabolism, but in most other animals, urate is degraded to allantoin by the enzyme uricase.



The role of HPRT in the purine salvage pathway

Priming DNA Synthesis

DNA polymerase cannot initiate new strands of nucleic acid synthesis because it can only add a nucleotide onto a pre-existing 3′-OH. Therefore, an 11 to 12 base-pair length of RNA (an RNA primer) is made at the beginning of each new strand of DNA.



Since the leading strand is synthesized as a single piece, there is only one RNA primer at the origin. On the lagging strand, each Okazaki fragment begins with a single RNA primer. DNA polymerase then makes DNA starting from each RNA primer. At the origin, a protein called **PriA** displaces the SSB proteins so a special RNA polymerase, called primase (DnaG), can enter and synthesize short RNA primers using ribonucleotides. Two molecules of DNA polymerase III bind to the primers on the leading and lagging strands and synthesize new DNA from the 3′ hydroxyls.

Primase, a special RNA polymerase, works with PriA to displace the SSB proteins and synthesize a short RNA primer at the origin. DNA polymerase then starts synthesis of the new DNA strand using the 3′-OH of the RNA primer. This synthesis occurs at multiple locations on the lagging strand.

Oxygenic photosynthesis

In plants, algae and cyanobacteria, photosynthesis releases oxygen. This is called oxygenic photosynthesis. Although there are some differences between oxygenic photosynthesis in plants, algae, and cyanobacteria, the overall process is quite similar in these organisms. Photosynthesis is not only needed by photosynthetic organism for energy but also for carbon fixation.

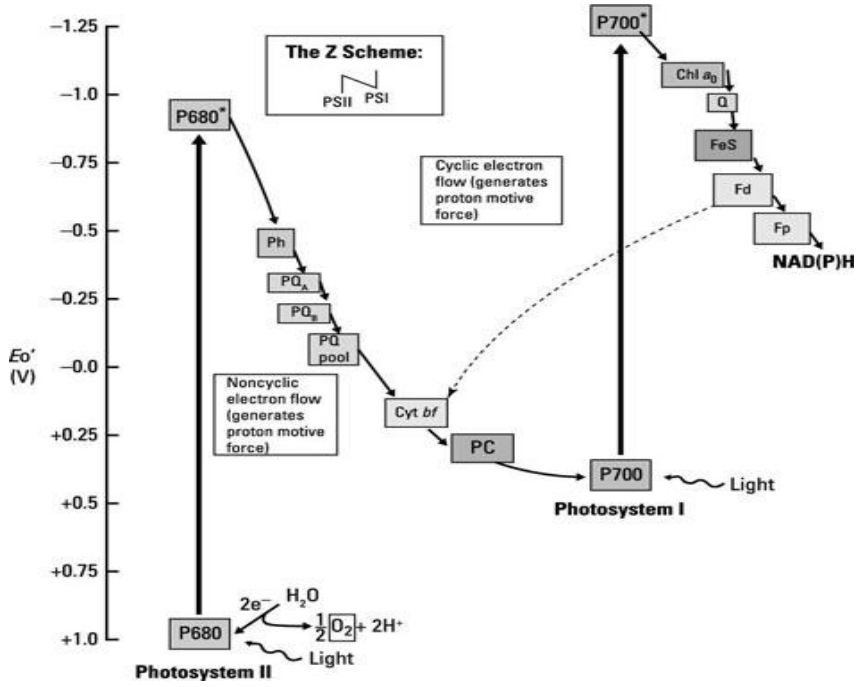


Figure: **Photosynthesis overview**: Photosynthesis changes sunlight into chemical energy, splits water to liberate O2, and fixes CO2 into sugar.

Carbon dioxide is converted into sugars in a process called carbon fixation. Carbon fixation is a redox reaction, so photosynthesis needs to supply both a source of energy to drive this process, and the electrons needed to convert carbon dioxide into a carbohydrate, which is a reduction reaction. In general outline, photosynthesis is the opposite of cellular respiration, where glucose and other compounds are oxidized to produce carbon dioxide, water, and release chemical energy. However, the two processes take place through a different sequence of chemical reactions and in different cellular compartments.

The general equation for photosynthesis is therefore:

$2n\ CO2 + 2n\ DH2 + photons \rightarrow 2(CH_2O)n + 2n\ DO$

Carbon dioxide + electron donor + light energy → carbohydrate + oxidized electron donor.

In oxygenic photosynthesis water is the electron donor and, since its hydrolysis releases oxygen, the equation for this process is:

$2n\ CO_2 + 4n\ H_2O + photons \rightarrow 2(CH_2O)n + 2n\ O_2 + 2n\ H_2O$

carbon dioxide + water + light energy → carbohydrate + oxygen + water

Often 2n water molecules are cancelled on both sides, yielding:

$2n\ CO_2 + 2n\ H_2O + photons \rightarrow 2(CH_2O)n + 2n\ O_2$

carbon dioxide + water + light energy → carbohydrate + oxygen

In plants, algae and cyanobacteria, photosynthesis releases oxygen. This is called oxygenic photosynthesis. Although there are some differences between oxygenic photosynthesis in plants, algae, and cyanobacteria, the overall process is quite similar in these organisms.

Anoxygenic photosynthesis: Phototrophy is the process by which organisms trap light energy (photons) and store it as chemical energy in the form of ATP and/or reducing power in NADPH. There are two major types of phototrophy: chlorophyll-based chlorophototrophy and rhodopsin-based retinalophototrophy. Chlorophototrophy can further be divided into oxygenic photosynthesis and anoxygenic phototrophy. Oxygenic and anoxygenic photosynthesizing organisms undergo different reactions either in the presence of light or with no direct contribution of light to the chemical reaction (colloquially called “light reactions” and “dark reactions”, respectively).

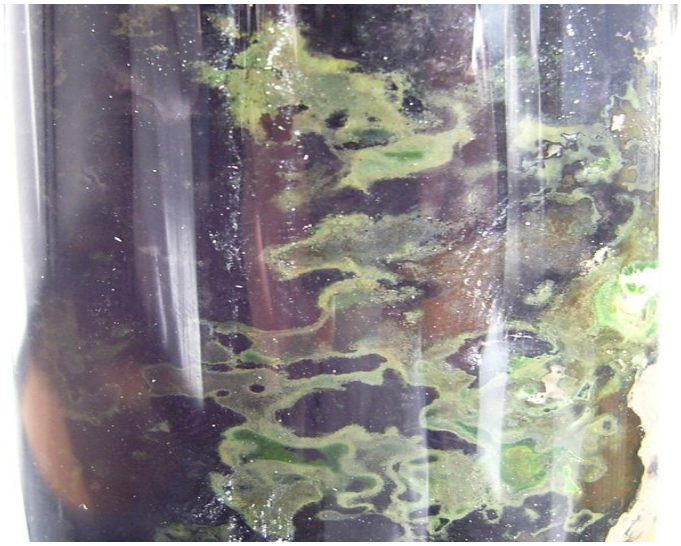


Figure: **Green d winogradsky**: A column containing green sulfur bacteria which uses anoxygenic photosynthesis.

Anoxygenic photosynthesis is the phototrophic process where light energy is captured and converted to ATP, without the production of oxygen. Water is therefore not used as an electron donor. There are several groups of bacteria that undergo anoxygenic photosynthesis: Green sulfur bacteria, green and red filamentous anoxygenic phototrophs (FAPs), phototrophic purple bacteria, phototrophic Acidobacteria, and phototrophic heliobacteria. Anoxygenic phototrophs have photosynthetic pigments called bacteriochlorophylls (similar to chlorophyll found in eukaryotes). Bacteriochlorophyll a and b have wavelengths of maximum absorption at 775 nm and 790 nm, respectively in ether. In vivo however, due to shared extended resonance structures, these pigments were found to maximally absorb wavelengths out further into the near-infrared. Bacteriochlorophylls c-g have the corresponding "peak" absorbance at more blue wavelengths when dissolved in an organic solvent, but are similarly red-shifted within their natural environment (with the exception of bacteriochlorophyll f, which has not been naturally observed).Unlike oxygenic phototrophs, anoxygenic photosynthesis only functions using (by phylum) either one of two possible types of photosystem. This restricts them to cyclic electron flow and are therefore unable to produce O_2 from the oxidization of H_2O.

The cyclic nature of the electron flow is typified in purple non-sulfur bacteria. The electron transport chain of purple non-sulfur bacteria begins when the reaction centre bacteriochlorophyll pair, P870, becomes excited from the absorption of light. Excited P870 will then donate an electron to Bacteriopheophytin, which then passes it on to a series of

electron carriers down the electron chain. In the process, it will generate a proton motor force (PMF) which can then be used to synthesize ATP by oxidative phosphorylation. The electron returns to P870 at the end of the chain so it can be used again once light excites the reaction-center. Therefore electrons are not left over to oxidize H_2O into O_2.

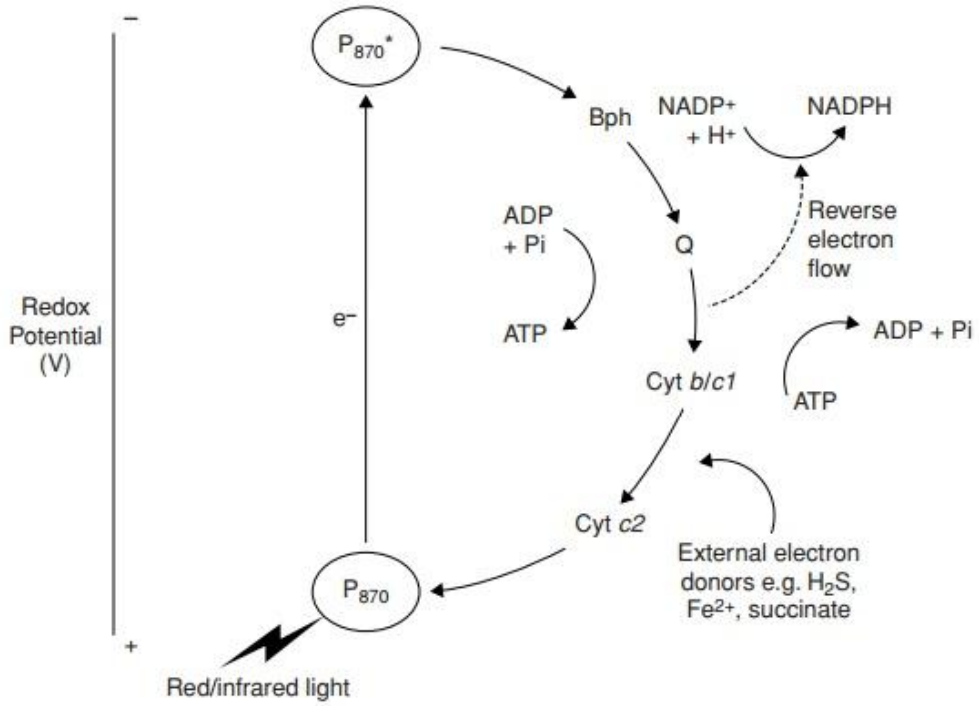
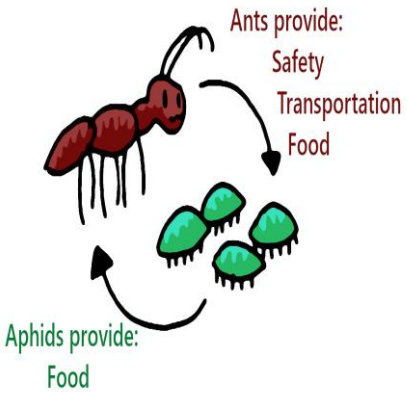


Figure 6.33 Electron flow in the anoxygenic photosynthesis of a purple bacterium. ATP is generated by the passage of electrons down an electron transport chain back to the reaction centre bacteriochlorophyll. Anoxygenic photosynthetic bacteria use molecules such as sulphur and hydrogen sulphide instead of water as external electron donors, hence no oxygen is generated. NADPH for use in CO_2 fixation must be generated by reverse electron flow. Bph = bacteriophaeophytin (bacteriochlorophyll *a* minus its magnesium atom)

Microbial interaction

Microorganisms can associate physically with other organismsin a variety of ways. One organism can be located on the surfaceof another, as an ectosymbiont. In this case, the ectosymbiontusually is a smaller organism located on the surface of a largerorganism. In contrast, one organism can be located within anotherorganism as an endosymbiont. While the simplest microbialinteractions involve two members, a symbiont and its host,a number of interesting organisms host more than one symbiont.The term consortium can be used to describe this physical relationship. For example, Thiothrix species, a sulfur-using bacterium,is attached to the surface of a mayfly larva and itself contains a parasitic bacterium. Fungi associated with plant root (mycorrhizal fungi) often contain endosymbiotic bacteria, aswell as having bacteria living on their surfaces. These physical associations can be intermittent and cyclic orpermanent. Examples of intermittent and cyclic associations ofmicroorganisms with plants and marine animals . Important human diseases, including listeriosis,malaria, leptospirosis, legionellosis, and vaginosis also involvesuch intermittent and cyclic symbioses. Interesting permanent relationshipsalso occur between bacteria and animals, as shown in table 30.2. Hosts include squid, leeches, aphids, nematodes, and mollusks.In each of these cases, an important characteristic of the host animalis conferred by the permanent bacterial symbiont. These interactions are now discussed



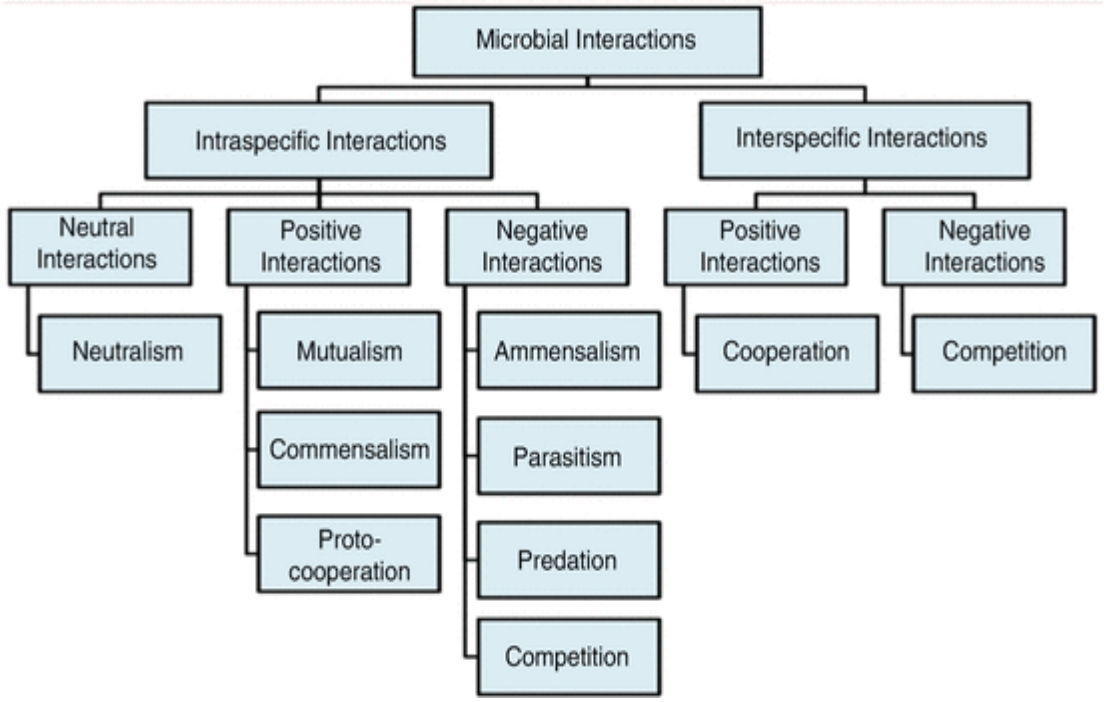
Mutualism: defines the relationship in which some reciprocal benefit accrues to bothpartners. This is an obligatory relationship in which the mutualistand the host are dependent on each other. When separated, inmany cases, the individual organisms will not survive. Several examples of mutualism are presented next. Microorganism-Insect Mutualisms: Mutualistic associations are common in the insects. This is relatedto the foods used by insects, which often include plant sapor animal fluids lacking in essential vitamins and amino acids.The required vitamins and amino acids are provided by bacterialsymbionts in exchange for a secure habitat and ample nutrients. The aphid is an excellentexample of this mutualistic relationship. This insect harbors the -proteobacterium Buchnera aphidicola in its

cytoplasm,and a mature insect contains literally millions of these bacteria inits body. The Buchnera provides its host with 10 essential aminoacids, and if the insect is treated with antibiotics, it dies. Likewise,B. aphidicola is an obligate mutualistic symbiont. The inabilityof either partner to grow without the other indicates thatthe two organisms demonstrate coevolution, or have evolved together.It is estimated that the B. aphidicola-aphid endosymbiosiswas established about 150 million years ago. The genomes oftwo different B. aphidicola strains have been sequenced and annotatedto reveal extreme genomic stability. These strains diverged50 to 70 million years ago, and since that time there have been no gene duplications, translocations, inversions, or genesacquired by horizontal transfer. The genomes are small, only0.64 Mb each with 93% of their genes common to both strains.

Furthermore, only two genes have no orthologs in their close relative *E. coli*. This tremendous degree of stability implies that althoughthe initial acquisition of the endosymbiont by ancestralaphids enabled their use of an otherwise deficient food source(sap), the bacteria have not continued to expand the ecologicalniche of their insect host through the acquisition of new traits that might be advantageous. The protozoan-termite relationship is another classic exampleof mutualism in which the flagellated protozoa live in the gut oftermites and wood roaches .These flagellates existon a diet of carbohydrates, acquired as cellulose ingested by theirhost The protozoa engulf wood particles, digest thecellulose, and metabolize it to acetate and other products. Termitesoxidize the acetate released by their flagellates. Because the hostis almost always incapable of synthesizing cellulases (enzymesthat catalyse the hydrolysis of cellulose), it is dependent on the 43 mutualisticprotozoa for its existence. This mutualistic relationshipcan be readily tested in the laboratory if wood roaches are placedin a bell jar containing wood chips and a high concentration of O2.Because O2 is toxic to the flagellates, they die. The wood roachesare unaffected by the high O2 concentration and continue to ingestwood, but they soon die of starvation due to a lack of cellulases.

Cooperation: Cooperation and commensalism are two positive but not obligatory types of symbioses found widely in the microbial world. These involve syntrophic relationships. Syntrophism is an association in which the growth of one organism either depends on or is improved by growth factors, nutrients, or substrates provided by another organismgrowing nearby. Sometimes both organisms benefit. Cooperation benefits both organisms . Acooperative relationship is not obligatory and, for most microbial ecologists, this nonobligatory aspect differentiates cooperation from mutualism. Unfortunately, it is often difficult to distinguish obligatory from nonobligatory because 50 that which is obligatory in

one habitat may not be in another (e.g., the laboratory). Nonetheless, the most useful distinction between cooperation and mutualism is the observation that cooperating organisms can be separated from one another and remain viable, although they may not function as well. Two examples of a cooperative relationship include the association between Desulfovibrio and Chromatium ,in which the carbon and sulfur cycles are linked, and the interactionof a nitrogen-fixing microorganism with a cellulolytic organism such as Cellulomonas . In the second example, the cellulose-degrading microorganism liberates glucose from the cellulose, which can be used by nitrogen-fixing microbes.



Microbial Interactions and Plant Growth

An excellent example of a cooperative biodegradative association is shown in figure 30.8. In this case degradation of the toxin 3-chlorobenzoate depends on the functioning of microorganisms with complementary capabilities. If any one of the three microorganisms is not present and active, the degradation of the substrate will not occur. This example points out how the sum of the microbes in a community can be considered greater than the contribution made by any single microorganism. In other cooperative relations, sulfide-dependent autotrophic filamentous microorganisms fix carbon dioxide and synthesize organic matter that serves as a carbon and energy source for a heterotrophic organism. Some of the most interesting include thepolychaete worms Alvinella pompejana, the Pompeii worm, and also Paralvinella palmiformis, the Palm worm. Both have filamentous bacteria on their dorsal

surfaces. These filamentous bacteria can tolerate high levels of metals such as arsenic, cadmium, and copper. When growing on the surface of the animal, they may provide protection from these toxic metals, as well as thermal protection; in addition, they appear to be used as a food source. A deep-sea crustacean has been discovered that usessulfur-oxidizing autotrophic bacteria as its food source. Thisshrimp, Rimicaris exoculata has filamentoussulfur-oxidizing bacteria growing on its surface .When these are dislodged the shrimp ingests them. This nominally "blind" shrimp use a reflective organ to respond to the glow emitted by geothermally active black smoker chimneys. The organ is sensitive to a light wavelength that is not detectable by humans. Another interesting example of bacterial epigrowth is shown by nematodes, including Eubostrichus parasitiferus, that live at the interface between oxic and anoxic sulfide-containing marine sediments. These animals are covered by sulfide-oxidizing bacteria that are present in intricate patterns .The bacteria not only decrease levels of toxic sulfide, which often surround the nematodes, but they also serve as a food supply. In 1990, hydrothermal vents were discovered in a freshwater environment at the bottom of Lake Baikal, the oldest (25 million years old) and deepest lake in the world. This lake is located in the far east of Russia and has the largest volume of any freshwater lake (not the largest area—which is Lake Superior). Microbial mats featuring long, white strands are in the center of the vent field where the highest temperatures are found. At the edge of the vent field, where the water temperature is lower, the microbial mats end, and sponges, gastropods, and other 51 organisms, which use the sulfur-oxidizing bacteria as a food source, are present .Similar although less developed areas have been found in Yellowstone Lake, Wyoming. A form of cooperation also occurs when a population of similar microorganisms monitors its own density—the process of quorum sensing. The microorganisms produce specific autoinducer compounds, and as the population increases and the concentration of these compounds reaches critical levels, specific genes are expressed.These responses are important for microorganisms that form associations with each other, plants, and animals. Intercellular communication is critical for the establishment of biofilms and for colonization of hosts by pathogens.

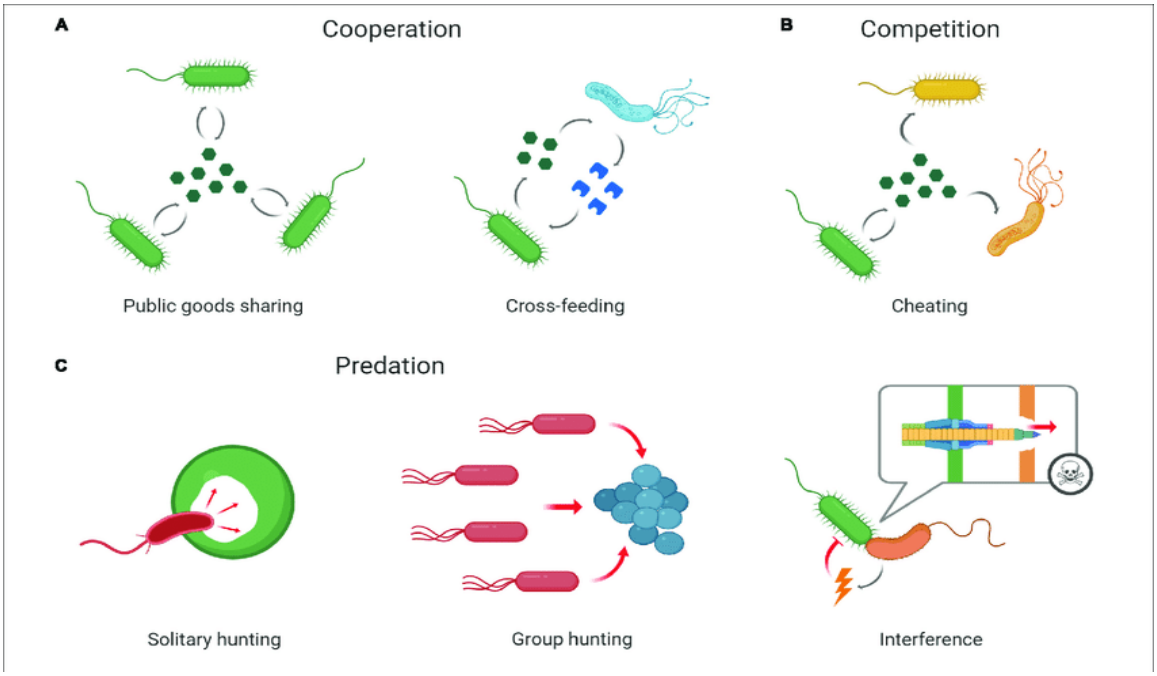
Commensalism: is a relationship in which one symbiont, the commensal, benefits while the other (sometimes called the host) is neither harmed nor helped, as shown in figure 30.1. This is a unidirectional process. Often both the host and the commensal "eat at the same table."

The spatial proximity of the two partners permits the commensal to feed on substances captured or ingested by the host, and the commensal often obtains shelter by living either on or in the host. The commensal is not directly dependent on the host metabolically and causes it no particular harm. When the commensal is separated from its host experimentally, it can survive without the addition of factors of host origin. Commensalistic relationships between microorganisms include situations in which the waste product of one microorganism is a substrate for another species. One good example is nitrification, the oxidation of ammonium ion to nitrite by microorganisms such as Nitrosomonas, and the subsequent oxidation of the nitrite to nitrate by Nitrobacter and similar bacteria. Nitrobacter benefits from its association with Nitrosomonas because it uses nitrite to obtain energy for growth. A second example of this type of relationship is found in anoxic methanogenic ecosystems such as sludge digesters, anoxic freshwater aquatic sediments, and flooded soils. In these environments, fatty acids can be degraded to produce H2 and methane by the interaction of two different bacterial groups. Methane production by methanogens depends on interspecies hydrogen transfer. A fermentative bacterium generates hydrogen gas, and the methanogen uses it quickly as a substrate for methane gas production. Various fermentative bacteria produce low molecular weight fatty acids that can be degraded by anaerobic bacteria such as Syntrophobacter to produce H2 as follows: Propionic acid → acetate + CO2+ H2 Syntrophobacter uses protons (H+ + H +→H2) as terminal electron acceptors in ATP synthesis. The bacterium gains sufficient energy for growth only when the H2 it generates is consumed. The products H2 and CO2 are used by methanogenic archaea such as Methanospirillum as follows: 4H2+ CO2 → CH4+ 2H2O By synthesizing methane, Methanospirillum maintains a low H2concentration in the immediate environment of both microbes. Continuous removal of H2 promotes further fatty acid fermentation and H2 production. Because increased H2 production and consumption stimulate the growth rates of Syntrophobacter andMethanospirillum, both participants in the relationship benefit. Commensalistic associations also occur when one microbial group modifies the environment to make it more suited for another organism. For example, common, nonpathogenic strains of Escherichiacoli live in the human colon, but also grow quite well outside the host, and thus are typical commensals.

When oxygenis used up by facultatively anaerobic E. coli, obligate anaerobes 55 such as Bacteroides are able to grow in the colon. The anaerobes benefit from their association with the host and E. coli, but E. coliderives no obvious benefit from the anaerobes. In this case the commensal E. coli contributes to the welfare of other symbionts. Commensalism can involve other environmental modifications. The synthesis of acidic waste products during fermentation stimulate the proliferation of more acid-tolerant microorganisms, which are only a minor part of the microbial community at neutral pH. A good example is the succession of microorganisms during milk spoilage. Biofilm formation provides another example. The colonization of a newly exposed surface by one type of microorganism (an initial colonizer) makes it possible for other microorganisms to attach to the microbially modified surface. Commensalism also is important in the colonization of the human body and the surfaces of other animals and plants. The microorganisms associated with an animal's skin and body orifices can use volatile, soluble, and particulate organic compounds from the host as nutrients. Under most conditions these microbes do not cause harm, other than possibly contributing to body odor. Sometimes when the host organism is stressed or the skin is punctured, these normally commensal microorganisms may become pathogenic by entering a different environment. Predation: As is the case with larger organisms, predation among microbes involves a predator species that attacks and usually kills its prey. Over the last several decades, microbiologists have discovered a number of fascinating bacteria that survive by their ability to prey upon other microbes. Several of the best examples are Bdellovibrio,Vampirococcus, and Daptobacter (figure 30.13). Bdellovibrio is an active hunter that is vigorously motile, swimming about looking for susceptible gram-negative bacterial prey. Upon sensing such a cell, Bdellovibrio swims faster until it collides with the prey cell. It then bores a hole through the outer membrane of its prey and enters the periplasmic space. As it grows, it forms a long filament that eventually septates to produce progeny bacteria. Lysis of the prey cell releases new Bdellovibrio cells. Bdellovibrio will not attack mammalian cells, and gram-negative prey bacteria have never been observed to acquire resistance to Bdellovibrio attack. This has raised interest in the use of Bdellovibrio as a "probiotic" to treat infected wounds. Although this is has not yet been tried, one can imaginethat with the rise in antibiotic-resistant pathogens, such forms of treatments may become viable alternatives.

Although Vampirococcus and Daptobacter also kill their prey, they gain entry in a less-dramatic fashion. Vampirococcusattaches itself as an epibiont to the outer membrane of its prey. It then secretes degradative enzymes that result in the release of the prey's cytoplasmic contents. In contrast, Daptobacter penetrates the prey cell and consumes the cytoplasmic contents directly. A surprising finding is that predation has many beneficial effects, especially when one considers interactive populations of predators and prey, as summarized in table 30.3. Simple ingestion and assimilation of a prey bacterium can lead to increased rates ofnutrient cycling, critical for the functioning of the microbial loop. In this process, organic matter produced through photosynthetic and chemotrophic activity is mineralized before it reaches higher consumers, allowing the minerals to be made available to the primary producers. Ingestion and 56 short-term retention of bacteria also are critical for ciliate functioning in the rumen, where methanogenic bacteria contribute to the health of the ciliates by decreasing toxic hydrogen levels by using H2 to produce methane, which then is passed from the rumen. Predation also can provide a protective, high-nutrient environment for particular prey. Ciliates ingest the gram-positive bacterium Legionella and protect this important pathogen from chlorine, which often is used in an attempt to control Legionellain cooling towers and air-conditioning units. The ciliate serves as a reservoir host. Legionella pneumophila also has been found to have a greater potential to invade macrophages and epithelial cells after predation, indicating that ingestion not only provides protection but also may enhance pathogenicity. A similar phenomenon of survival in protozoa has been observed for Mycobacteriumavium, a pathogen of worldwide concern. These protective aspects of predation have major implications for survival and control of disease-causing microorganisms in the biofilms present in water supplies and air-conditioning systems. Fungi often show interesting predatory skills. Some fungi can trap protozoa by the use of sticky hyphae or knobs, sticky networks of hyphae, or constricting or nonconstricting rings. Aclassic example is Arthrobotrys, which traps nematodes by use of constricting rings. After the nematode is trapped, hyphae grow into the immobilized prey and the cytoplasm is used as a nutrient. Other fungi have conidia that, after ingestion by an unsuspectingpredator, grow and attack the susceptible host from inside the intestinaltract. In this situation the fungus penetrates the host cellsin a complex interactive process.

Clearly predation in the microbial world is not straightforward. It often has a fatal and final outcome for an individual prey organism but it can have a wide range of beneficial effects on prey populations. Predation is clearly critical in the functioning of natural environments.



Social interactions among microbes

Parasitism: Parasitism is one of the most complex microbial interactions; the line between parasitism and predation is difficult to define . This is a relationship between two organisms in which one benefits from the other, and the host is usually harmed. This can involve 57 nutrient acquisition and/or physical maintenance in or on the host. In parasitism there is always some co-existence between host and parasite. Successful parasites have evolved to co-exist in equilibrium with their hosts. This is because a host that dies immediately after parasite invasion may prevent the microbe from reproducing to sufficient numbers to ensure colonization of a new host. But what happens if the host-parasite equilibrium is upset? If the balance favors the host (perhaps by a strong immune defense orantimicrobial therapy), the parasite loses its habitat and may be unable to survive. On the other hand, if the equilibrium is shifted to favor the parasite, the host becomes ill, and depending on the specific host-parasite relationship, may die.

One good example is the disease typhus. This disease is caused by the rickettsia Rickettsiatyphi, which is harbored in fleas that live on rats. It is transmitted to humans who are bitten by such fleas, so in order to contract typhus, one must be in close proximity to rats. Humans often live in association with rats, and in such communities there is always a small number of people with typhus—that is to say, typhus is endemic. However, during times of war or when people are forced to become refugees, lack of sanitation and overcrowding result in an increased number of rat-human interactions. Typhus can then reach epidemic proportions. During the Crimean War (1853–1856), about 213,000 men were killed or wounded in combat while over 850,000 were sickened or killed by typhus. On the other hand, a controlled parasite-host relationship can be maintained for long periods of time. For example, lichens are the association between specific ascomycetes (a fungus) and certain genera of either green algae or cyanobacteria. In lichen, the fungal partner is termed the mycobiont and the algal or cyanobacterial partner, the phycobiont. In the past the lichen symbiosis was considered to be a mutualistic interaction. It recently has been found that lichen forms only when the two potential partners are nutritionally deprived. In nutrient-limited environments, the relationship between the fungus and its photosynthetic partner has coevolved to the point where lichen morphology and metabolic relationships are extremely stable. In fact, lichens are assigned generic and species names. The characteristic morphology of a given lichen is a property of the association and is not exhibited by either symbiont individually.Because the phycobiont is a photoautotroph—dependent only on light, carbon dioxide, and mineral nutrients—the fungus can get its organic carbon directly from the alga or cyanobacterium. The fungus often obtains nutrients from its partner by projections of fungal hyphae called haustoria, which penetrate the phycobiont cell wall. It also uses the O_2 produced during phycobiont photophosphorylation in carrying out respiration. In turn the fungus protects the phycobiont from high light intensities, provideswater and minerals to it, and creates a firm substratum within which the phycobiont can grow protected from environmental stress. The invasive nature of the fungal partner is why lichens are considered parasitic relationships. An important aspect of many symbiotic relationships, including parasitism, is that over time, the symbiont, once it has established a relationship with the host, will tend to discard excess, unused genomic information, a process called genomic reduction.

This is clearly the case with the aphid endosymbiont Buchneraaphidicola and it has also occurred with the parasite Mycobacterium leprae, and with the microsporidium Encephalitozooncuniculi. The latter 58 organism, which parasitizes a wide range of animals, including humans, now can only survive inside the host cell.

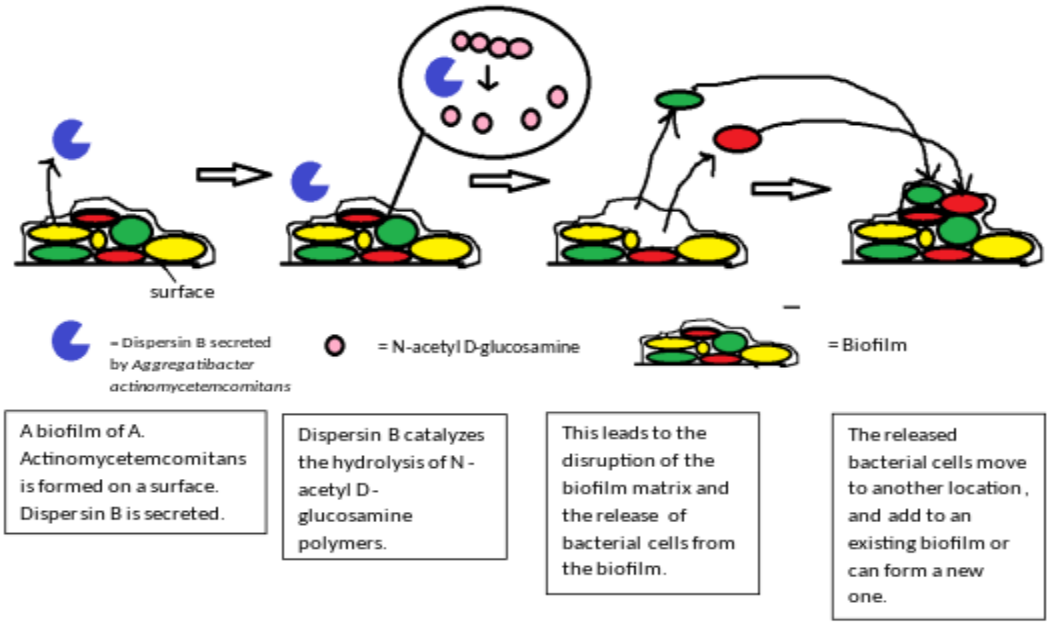
Amensalism: It describes he adverse effect that one organism has on another organism .This is a unidirectional process based on the release of a specific compound by one organism which has a negative effect on another organism. A classic example of amensalism is the production of antibiotics that can inhibit or kill a susceptible microorganism .Community complexity is demonstrated by the capacity of attine ants (ants belonging to a New World tribe) to take advantage of an amensalistic relationship between an actinomycete and the parasitic fungi Escovopsis. This amensalistic relationship enables the ant to maintain a mutualism with another fungal species, Leucocoprini. Amazingly, these ants cultivate a garden of Leucocoprini for their own nourishment. To prevent the parasitic fungus Escovopsis from decimating their fungal garden, the ants also promote the growth of an actinomycete of the genus Pseudonocardia, which produces an antimicrobial compound that inhibits the growth of Escovopsis. This unique amensalistic process appears to have evolved 50 to 65million years ago in South America. Thus this relationship has been subject to millions of years of coevolution, such that particular groups of ants cultivate specific strains of fungi that are then subject to different groups of Escovopsis parasites. In addition, the ants have developed intricate crypts within their exoskeletons for the growth of the antibiotic-producing Pseudonocardia, these crypts have been modified throughout the ants' evolutionary history. The most primitive "paleo-attine" ants carry the bacterium on their forelegs, "lower" and "higher" attines have evolved special plates on their ventral surfaces, while the entire surface of the most recent attines, leaf-cutter ants of the genus Acromyrmex, are covered with the bacterium. Related ants that do not cultivate fungal gardens (e.g., Atta sp.) do not host Pseudonocardia. This unique multipartner relationship has enabled scientists to explore the behavioral, physiological, and structural aspects of the organisms involved. Other important amensalistic relationships involve microbial production of specific organic compounds that disrupt cell wall orplasma membrane integrity of target microorganisms. These include the bacteriocins. The bacteriocin nisin has been used as an additive for controlling the growth of undesired pathogens in dairy products for over 40 years. Antibacterial peptides also can be released by the host in the intestine and other sites.

These molecules, called cecropins in insects and defensins in mammals, are effector molecules that play significant roles in innate immunity. In vertebrates these molecules are released by phagocytes and intestinal cells, and have powerful antimicrobial activity. Human sweat is also antimicrobial. Sweat glands produce an antimicrobial peptide called dermicidin. The skin also produces similar compounds including an antimicrobial peptide called cathelicidin.Finally, metabolic products, such as organic acids formed in fermentation, can produce amensalistic effects. These compounds inhibit growth by changing the environmental pH, for example, during natural milk spoilage.

Competition: Competition arises when different organisms within a population or community try to acquire the same resource, whether this is a physical location or a particular limiting nutrient . If one of the two competing organisms can dominate the environment, whether by occupying the physical habitat or by consuming a limiting nutrient, it will outgrow the other organism. This phenomenon was studied by E. F. Gause, who in 1934 described it as the competitive exclusion principle. He found that if two competingciliates overlapped too much in terms of their resource use, oneof the two protozoan populations was excluded. In chemostats,competition for a limiting nutrient may occur among microorganisms with transport systems of differing affinity. This can lead to the exclusion of the slower-growing population under a particular set of conditions. If the dilution rate is changed, the previously slowergrowing population may become dominant. Often two microbial populations that appear to be similar nevertheless coexist. In this case, they share the limiting resource (space, a limiting nutrient) and coexist while surviving at lower population levels.

Biofilm: Although scientists observed as early as the 1940s that more microbes in aquatic environments were found attached to surfaces (sessile) rather than were free-floating (planktonic), only relativelyrecently has this fact gained the attention of microbiologists. These attached microbes are members of complex, slime-encased communities called biofilms. Biofilms are ubiquitous in nature. There they are most often seen as layers of slime on rocks or other objects in water. When they form on the hulls of (a) (b)boats and ships, they cause corrosion, which limits the life of the ships and results in economic losses. Of major concern is the formation of biofilms on medical devices such as hip and knee implants .These biofilms often cause serious illness and failure of the medical device. Biofilm formation is apparently an ancient ability among the procaryotes, as evidence for biofilms can be found in the fossil record from about 3.4 billion years ago.

Biofilms can form on virtually any surface, once it has been conditioned by proteins and other molecules present in the environment .Microbes reversibly attach to the conditioned surface and eventually begin releasing polysaccharides, proteins, and DNA. These polymers allow the microbes to stick more stably to the surface. As the biofilm thickens and matures, the microbes reproduce and secrete additional polymers. The end result is a complex, dynamic community of microorganisms. The microbes interact in a variety of ways. For instance, the waste products of one microbe may be the energy source for another microbe. The cells also communicate with each other as described next. Finally, the presence of DNA in the extracellular slime can be taken up by members of the biofilm community. Thus genes can be transferred from one cell (or species) to another. While in the biofilm, microbes are protected from numerous harmful agents such as UV light, antibiotics, and other antimicrobial agents. This is due in part to the extracellular matrix in which they are embedded, but it also is due to physiological changes. Indeed, numerous proteins synthesized or activated in biofilm cells are not observed in planktonic cells and vice versa. The resistance of biofilm cells to antimicrobial agents has serious consequences. When biofilms form on a medical device such as a hip implant , they are difficult to kill and can cause serious illness. Often the only way to treat patients in this situation is by removing the implant. Another problem with biofilms is that cells are regularly sloughed off. This can have many consequences. For instance, biofilms in a city's water distribution pipes can serve as a source of contaminationafter the water leaves a water treatment facility.



Biofilm

Formation of biofilms

The formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. The first colonist bacteria of a biofilm may adhere to the surface initially by the weak van der Waals forces and hydrophobic effects. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili. Unique groups of Archaea that inhabit anoxic groundwater have similar structures called hami. Each hamus is a long tube with three hook attachments that are used to attach to each other or to a surface, enabling a community to develop. Hyperthermophilic archaeon Pyrobaculum calidifontis produce bundling pili which are homologous to the bacterial TasA filaments, a major component of the extracellular matrix in bacterial biofilms, which contribute to biofilm stability. TasA homologs are encoded by many other archaea, suggesting mechanistic similarities and evolutionary connection between bacterial and archaeal biofilms.

Biofilm of golden hydrophobic bacteria; ceiling of Golden Dome Cave, a lava tube in Lava Beds National Monument Hydrophobicity can also affect the ability of bacteria to form biofilms. Bacteria with increased hydrophobicity have reduced repulsion between the substratum and the bacterium. Some bacteria species are not able to attach to a surface on their own successfully due to their limited motility but are instead able to anchor themselves to the matrix or directly to other, earlier bacteria colonists. Non-motile bacteria cannot recognize surfaces or aggregate together as easily as motile bacteria.

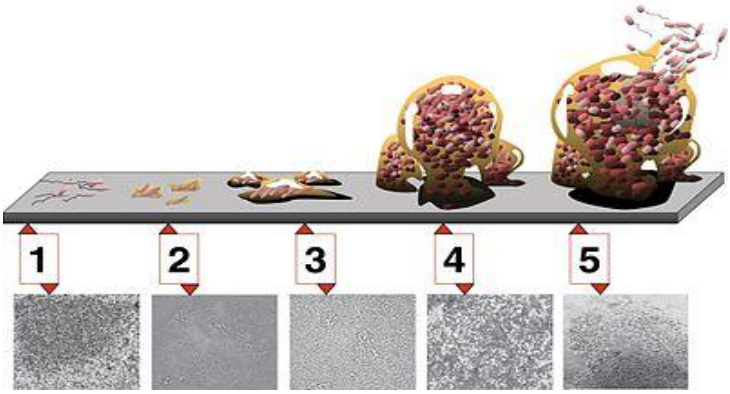
During surface colonization bacteria cells are able to communicate using quorum sensing (QS) products such as N-acyl homoserine lactone (AHL). Once colonization has begun, the biofilm grows by a combination of cell division and recruitment. Polysaccharide matrices typically enclose bacterial biofilms. The matrix exopolysaccharides can trap QS autoinducers within the biofilm to prevent predator detection and ensure bacterial survival. In addition to the polysaccharides, these matrices may also contain material from the surrounding environment, including but not limited to minerals, soil particles, and blood components, such as erythrocytes and fibrin. The final stage of biofilm formation is known as development, and is the stage in which the biofilm is established and may only change in shape and size.

The development of a biofilm may allow for an aggregate cell colony (or colonies) to be increasingly tolerant or resistant to antibiotics.

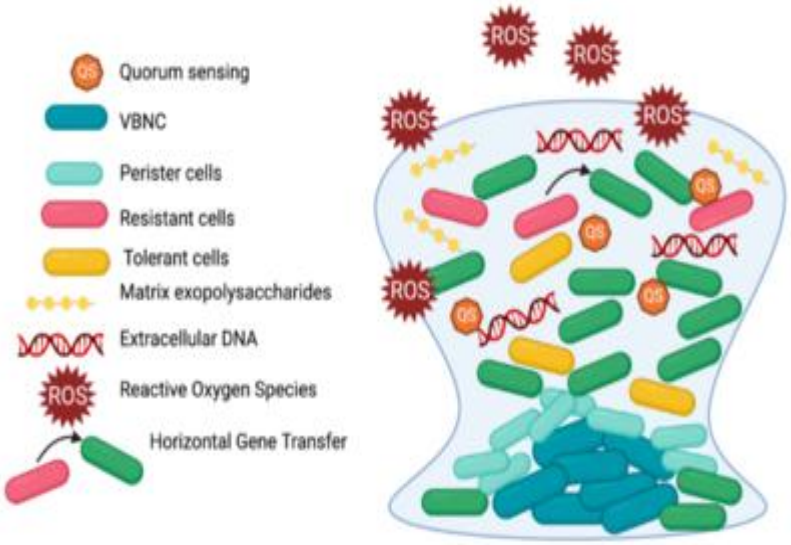
Cell-cell communication or quorum sensing has been shown to be involved in the formation of biofilm in several bacterial species.

Development

Biofilms are the product of a microbial developmental process. The process is summarized by five major stages of biofilm development, as shown in the diagram below



Five stages of biofilm development: (1) Initial attachment, (2) Irreversible attachment, (3) Maturation I, (4) Maturation II, and (5) Dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing P. aeruginosa biofilm.



Mature biofilm structure

Biofilm is characterised by heterogenous environment and the presence of a variety of subpopulations. A biofilm structure is composed of metabolically active (both resistant and tolerant) and non-active cells (viable but not culturable cells and persisters) as well as polymer matrix consisting of polysaccharide, extracellular DNA and proteins. Biofilm growth

is associated with an escalated level of mutations and horizontal gene transfer which is promoted in due to the packed and dense structure. Bacteria in biofilms communicate by quorum sensing, which activates genes participating in virulence factors production.

Dispersal:

Dispersal of cells from the biofilm colony is an essential stage of the biofilm life cycle. Dispersal enables biofilms to spread and colonize new surfaces. Enzymes that degrade the biofilm extracellular matrix, such as dispersin B and deoxyribonuclease, may contribute to biofilm dispersal. Enzymes that degrade the biofilm matrix may be useful as anti-biofilm agents. Evidence has shown that a fatty acid messenger, cis-2-decenoic acid, is capable of inducing dispersion and inhibiting growth of biofilm colonies. Secreted by Pseudomonas aeruginosa, this compound induces cyclo heteromorphic cells in several species of bacteria and the yeast Candida albicans. Nitric oxide has also been shown to trigger the dispersal of biofilms of several bacteria species at sub-toxic concentrations. Nitric oxide has potential as a treatment for patients that have chronic infections caused by biofilms.

It was generally assumed that cells dispersed from biofilms immediately go into the planktonic growth phase. However, studies have shown that the physiology of dispersed cells from Pseudomonas aeruginosa biofilms is highly different from that of planktonic and biofilm cells. Hence, the dispersal process is a unique stage during the transition from biofilm to planktonic lifestyle in bacteria. Dispersed cells are found to be highly virulent against macrophages and Caenorhabditis elegans, but highly sensitive towards iron stress, as compared with planktonic cells.

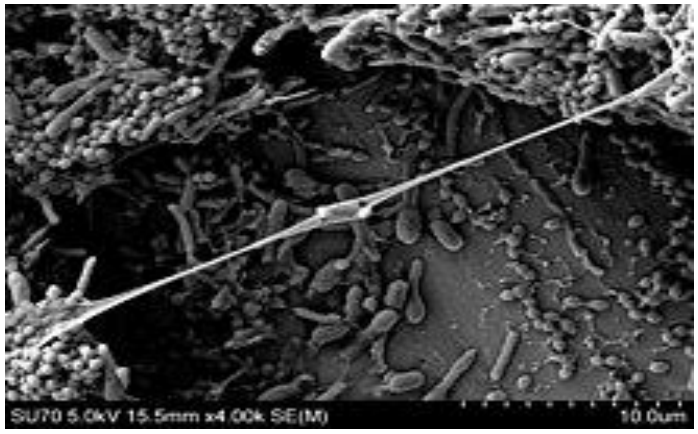
Furthermore, Pseudomonas aeruginosa biofilms undergo distinct spatiotemporal dynamics during biofilm dispersal or disassembly, with contrasting consequences in recolonization and disease dissemination. Biofilm dispersal induced bacteria to activate dispersal genes to actively depart from biofilms as single cells at consistent velocities but could not recolonize fresh surfaces. In contrast, biofilm disassembly by degradation of a biofilm exopolysaccharide released immotile aggregates at high initial velocities, enabling the bacteria to recolonize fresh surfaces and cause infections in the hosts efficiently. Hence, biofilm dispersal is more complex than previously thought, where bacterial populations adopting distinct behavior after biofilm departure may be the key to survival of bacterial species and dissemination of diseases.

Properties:

Biofilms are usually found on solid substrates submerged in or exposed to an aqueous solution, although they can form as floating mats on liquid surfaces and also on the surface of leaves, particularly in high humidity climates. Given sufficient resources for growth, a biofilm will quickly grow to be macroscopic (visible to the naked eye). Biofilms can contain many different types of microorganism, e.g. bacteria, archaea, protozoa, fungi and algae; each group performs specialized metabolic functions. However, some organisms will form single-species films under certain conditions. The social structure (cooperation/competition) within a biofilm depends highly on the different species present.

Extracellular matrix

The EPS matrix consists of exopolysaccharides, proteins and nucleic acids. A large proportion of the EPS is more or less strongly hydrated, however, hydrophobic EPS also occur; one example is cellulose which is produced by a range of microorganisms. This matrix encases the cells within it and facilitates communication among them through biochemical signals as well as gene exchange. The EPS matrix also traps extracellular enzymes and keeps them in close proximity to the cells. Thus, the matrix represents an external digestion system and allows for stable synergistic microconsortia of different species. Some biofilms have been found to contain water channels that help distribute nutrients and signalling molecules. This matrix is strong enough that under certain conditions, biofilms can become fossilized (stromatolites).



Scanning electron micrograph of mixed-culture biofilm, demonstrating in detail a spatially heterogeneous arrangement of bacterial cells and extracellular polymeric substances.

Bacteria living in a biofilm usually have significantly different properties from free-floating bacteria of the same species, as the dense and protected environment of the film allows them to cooperate and interact in various ways. One benefit of this environment is increased resistance to detergents and antibiotics, as the dense extracellular matrix and the outer layer of cells protect the interior of the community. In some cases antibiotic resistance can be increased up to 5,000 times. Lateral gene transfer is often facilitated within bacterial and archaeal biofilms and can leads to a more stable biofilm structure. Extracellular DNA is a major structural component of many different microbial biofilms. Enzymatic degradation of extracellular DNA can weaken the biofilm structure and release microbial cells from the surface.

However, biofilms are not always less susceptible to antibiotics. For instance, the biofilm form of Pseudomonas aeruginosa has no greater resistance to antimicrobials than do stationary-phase planktonic cells, although when the biofilm is compared to logarithmic-phase planktonic cells, the biofilm does have greater resistance to antimicrobials. This resistance to antibiotics in both stationary-phase cells and biofilms may be due to the presence of persister cells.

Habitats

Biofilms are ubiquitous in organic life. Nearly every species of microorganism have mechanisms by which they can adhere to surfaces and to each other. Biofilms will form on virtually every non-shedding surface in non-sterile aqueous or humid environments. Biofilms can grow in the most extreme environments: from, for example, the extremely hot, briny waters of hot springs ranging from very acidic to very alkaline, to frozen glaciers.

Biofilms can be found on rocks and pebbles at the bottoms of most streams or rivers and often form on the surfaces of stagnant pools of water. Biofilms are important components of food chains in rivers and streams and are grazed by the aquatic invertebrates upon which many fish feed. Biofilms are found on the surface of and inside plants. They can either contribute to crop disease or, as in the case of nitrogen-fixing rhizobia on root nodules, exist symbiotically with the plant. Examples of crop diseases related to biofilms include citrus canker, Pierce's disease of grapes, and bacterial spot of plants such as peppers and tomatoes.

Percolating filters

Percolating filters in sewage treatment works are highly effective removers of pollutants from settled sewage liquor. They work by trickling the liquid over a bed of hard material which is designed to have a very large surface area.

A complex biofilm develops on the surface of the medium which absorbs, adsorbs and metabolises the pollutants. The biofilm grows rapidly and when it becomes too thick to retain its grip on the media it washes off and is replaced by newly grown film. The washed off ("sloughed" off) film is settled out of the liquid stream to leave a highly purified effluent.

Slow sand filter

Slow sand filters are used in water purification for treating raw water to produce a potable product. They work through the formation of a biofilm called the hypogeal layer or Schmutzdecke in the top few millimetres of the fine sand layer. The Schmutzdecke is formed in the first 10–20 days of operation and consists of bacteria, fungi, protozoa, rotifera and a range of aquatic insect larvae. As an epigeal biofilm ages, more algae tend to develop and larger aquatic organisms may be present including some bryozoa, snails and annelid worms. The surface biofilm is the layer that provides the effective purification in potable water treatment, the underlying sand providing the support medium for this biological treatment layer. As water passes through the hypogeal layer, particles of foreign matter are trapped in the mucilaginous matrix and soluble organic material is adsorbed. The contaminants are metabolised by the bacteria, fungi and protozoa. The water produced from an exemplary slow sand filter is of excellent quality with 90–99% bacterial cell count reduction.

Rhizosphere

Plant-beneficial microbes can be categorized as plant growth-promoting rhizobacteria. These plant growth-promoters colonize the roots of plants, and provide a wide range of beneficial functions for their host including nitrogen fixation, pathogen suppression, anti-fungal properties, and the breakdown of organic materials. One of these functions is the defense against pathogenic, soil-borne bacteria and fungi by way of induced systemic resistance (ISR) or induced systemic responses triggered by pathogenic microbes (pathogen-induced systemic acquired resistance). Plant exudates act as chemical signals for host specific bacteria to colonize. Rhizobacteria colonization steps include attractions, recognition, adherence, colonization, and growth. Bacteria that have been shown to be beneficial and form biofilms include Bacillus, Pseudomonas, and Azospirillum. Biofilms in the rhizosphere often result in pathogen or plant induced systemic resistances. Molecular properties on the surface of the bacterium cause an immune response in the plant host. These microbe associated molecules interact with receptors on the surface of plant cells, and activate a biochemical response that is thought to include several different genes at a number of loci.

Several other signaling molecules have been linked to both induced systemic responses and pathogen-induced systemic responses, such as jasmonic acid and ethylene. Cell envelope components such as bacterial flagella and lipopolysaccharides, which are recognized by plant cells as components of pathogens. Certain iron metabolites produced by Pseudomonas have also been shown to create an induced systemic response. This function of the biofilm helps plants build stronger resistance to pathogens.

Plants that have been colonized by PGPR forming a biofilm have gained systemic resistances and are primed for defense against pathogens. This means that the genes necessary for the production of proteins that work towards defending the plant against pathogens have been expressed, and the plant has a "stockpile" of compounds to release to fight off pathogens. A primed defense system is much faster in responding to pathogen induced infection, and may be able to deflect pathogens before they are able to establish themselves. Plants increase the production of lignin, reinforcing cell walls and making it difficult for pathogens to penetrate into the cell, while also cutting off nutrients to already infected cells, effectively halting the invasion. They produce antimicrobial compounds such as phytoalexins, chitinases, and proteinase inhibitors, which prevent the growth of pathogens. These functions of disease suppression and pathogen resistance ultimately lead to an increase in agricultural production and a decrease in the use of chemical pesticides, herbicides, and fungicides because there is a reduced amount of crop loss due to disease. Induced systemic resistance and pathogen-induced systemic acquired resistance are both potential functions of biofilms in the rhizosphere, and should be taken into consideration when applied to new age agricultural practices because of their effect on disease suppression without the use of dangerous chemicals.

Mammalian gut

Studies in 2003 discovered that the immune system supports biofilm development in the large intestine. This was supported mainly with the fact that the two most abundantly produced molecules by the immune system also support biofilm production and are associated with the biofilms developed in the gut. This is especially important because the appendix holds a mass amount of these bacterial biofilms.This discovery helps to distinguish the possible function of the appendix and the idea that the appendix can help reinoculate the gut with good gut flora. However, modified or disrupted states of biofilms in the gut have been connected to diseases such as inflammatory bowel disease and colorectal cancer.

Human environment

In the human environment, biofilms can grow in showers very easily since they provide a moist and warm environment for them to thrive. They can form inside water and sewage pipes and cause clogging and corrosion. On floors and counters, they can make sanitation difficult in food preparation areas. In soil, they can cause bioclogging. In cooling- or heating-water systems, they are known to reduce heat transfer. Biofilms in marine engineering systems, such as pipelines of the offshore oil and gas industry, can lead to substantial corrosion problems. Corrosion is mainly due to abiotic factors; however, at least 20% of corrosion is caused by microorganisms that are attached to the metal subsurface (i.e., microbially influenced corrosion).

Ship fouling

Bacterial adhesion to boat hulls serves as the foundation for biofouling of seagoing vessels. Once a film of bacteria forms, it is easier for other marine organisms such as barnacles to attach. Such fouling can reduce maximum vessel speed by up to 20%, prolonging voyages and consuming fuel. Time in dry dock for refitting and repainting reduces the productivity of shipping assets, and the useful life of ships is also reduced due to corrosion and mechanical removal (scraping) of marine organisms from ships' hulls.

Stromatolites

Stromatolites are layered accretionary structures formed in shallow water by the trapping, binding and cementation of sedimentary grains by microbial biofilms, especially of cyanobacteria. Stromatolites include some of the most ancient records of life on Earth, and are still forming today.

Dental plaque

Within the human body, biofilms are present on the teeth as dental plaque, where they may cause tooth decay and gum disease. These biofilms can either be in an uncalcified state that can be removed by dental instruments, or a calcified state which is more difficult to remove. Removal techniques can also include antimicrobials.

Dental plaque is an oral biofilm that adheres to the teeth and consists of many species of both bacteria and fungi (such as Streptococcus mutans and Candida albicans), embedded in salivary polymers and microbial extracellular products. The accumulation of microorganisms subjects the teeth and gingival tissues to high concentrations of bacterial metabolites which results in dental disease.

Biofilm on the surface of teeth is frequently subject to oxidative stress and acid stress. Dietary carbohydrates can cause a dramatic decrease in pH in oral biofilms to values of 4 and below (acid stress). A pH of 4 at body temperature of 37°C causes depurination of DNA, leaving apurinic (AP) sites in DNA, especially loss of guanine.

Dental plaque biofilm can result in dental caries if it is allowed to develop over time. An ecologic shift away from balanced populations within the dental biofilm is driven by certain (cariogenic) microbiological populations beginning to dominate when the environment favors them. The shift to an acidogenic, aciduric, and cariogenic microbiological population develops and is maintained by frequent consumption of fermentable dietary carbohydrate. The resulting activity shift in the biofilm (and resulting acid production within the biofilm, at the tooth surface) is associated with an imbalance of demineralization over remineralization, leading to net mineral loss within dental hard tissues (enamel and then dentin), the symptom being a carious lesion, or cavity. By preventing the dental plaque biofilm from maturing or by returning it back to a non-cariogenic state, dental caries can be prevented and arrested. This can be achieved through the behavioral step of reducing the supply of fermentable carbohydrates (i.e. sugar intake) and frequent removal of the biofilm (i.e., toothbrushing).

Intercellular communication

A peptide pheromone quorum sensing signaling system in S. mutans includes the competence stimulating peptide (CSP) that controls genetic competence. Genetic competence is the ability of a cell to take up DNA released by another cell. Competence can lead to genetic transformation, a form of sexual interaction, favored under conditions of high cell density and/or stress where there is maximal opportunity for interaction between the competent cell and the DNA released from nearby donor cells. This system is optimally expressed when S. mutans cells reside in an actively growing biofilm. Biofilm grown S. mutans cells are genetically transformed at a rate 10- to 600-fold higher than S. mutans growing as free-floating planktonic cells suspended in liquid.

When the biofilm, containing S. mutans and related oral streptococci, is subjected to acid stress, the competence regulon is induced, leading to resistance to being killed by acid. As pointed out by Michod et al., transformation in bacterial pathogens likely provides for effective and efficient recombinational repair of DNA damages. It appears that S. mutans can survive the frequent acid stress in oral biofilms, in part, through the recombinational repair provided by competence and transformation.

Predator-prey interactions

Predator-prey interactions between biofilms and bacterivores, such as the soil-dwelling nematode Caenorhabditis elegans, had been extensively studied. Via the production of sticky matrix and formation of aggregates, Yersinia pestis biofilms can prevent feeding by obstructing the mouth of C. elegans. Moreover, Pseudomonas aeruginosa biofilms can impede the slithering motility of C. elegans, termed as 'quagmire phenotype', resulting in trapping of C. elegans within the biofilms and preventing the exploration of nematodes to feed on susceptible biofilms. This significantly reduced the ability of predator to feed and reproduce, thereby promoting the survival of biofilms. Pseudomonas aeruginosa biofilms can also mask their chemical signatures, where they reduced the diffusion of quorum sensing molecules into the environment and prevented the detection of C. elegans.

Taxonomic diversity

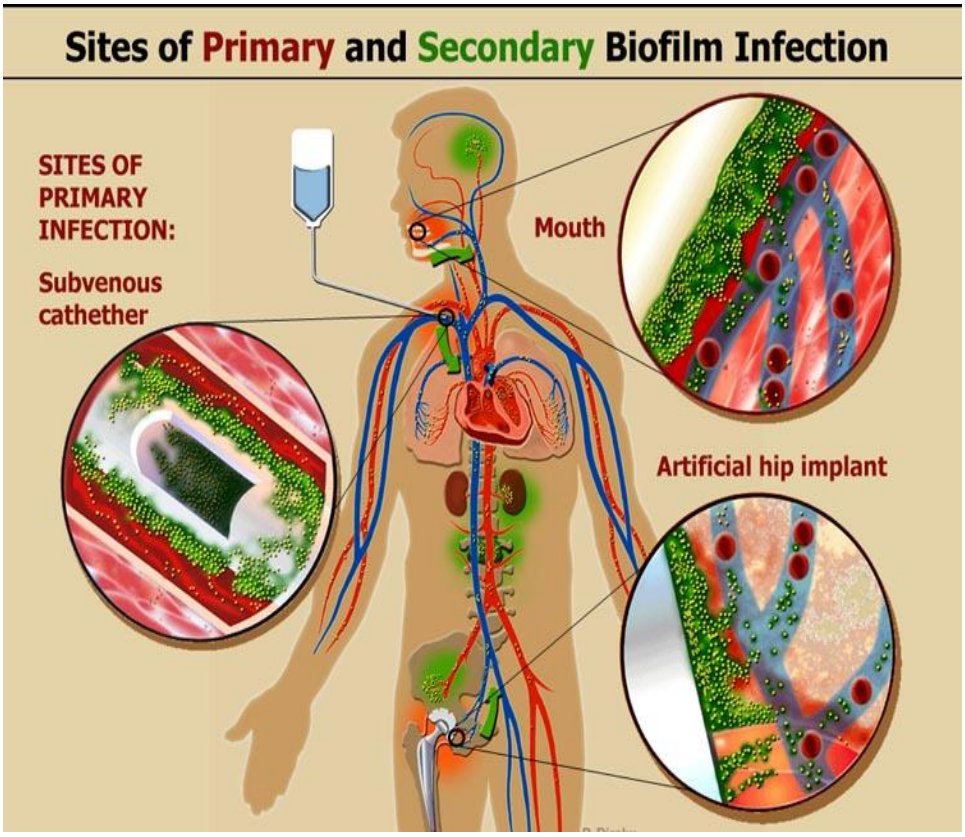
Many different bacteria form biofilms, including gram-positive (e.g. Bacillus spp, Listeria monocytogenes, Staphylococcus spp, and lactic acid bacteria, including Lactobacillus plantarum and Lactococcus lactis) and gram-negative species (e.g. Escherichia coli, or Pseudomonas aeruginosa). Cyanobacteria also form biofilms in aquatic environments.

Biofilms are formed by bacteria that colonize plants, e.g. Pseudomonas putida, Pseudomonas fluorescens, and related pseudomonads which are common plant-associated bacteria found on leaves, roots, and in the soil, and the majority of their natural isolates form biofilms. Several nitrogen-fixing symbionts of legumes such as Rhizobium leguminosarum and Sinorhizobium meliloti form biofilms on legume roots and other inert surfaces.

Along with bacteria, biofilms are also generated by archaea and by a range of eukaryotic organisms, including fungi e.g. Cryptococcus laurentii and microalgae. Among microalgae, one of the main progenitors of biofilms are diatoms, which colonise both fresh and marine environments worldwide.

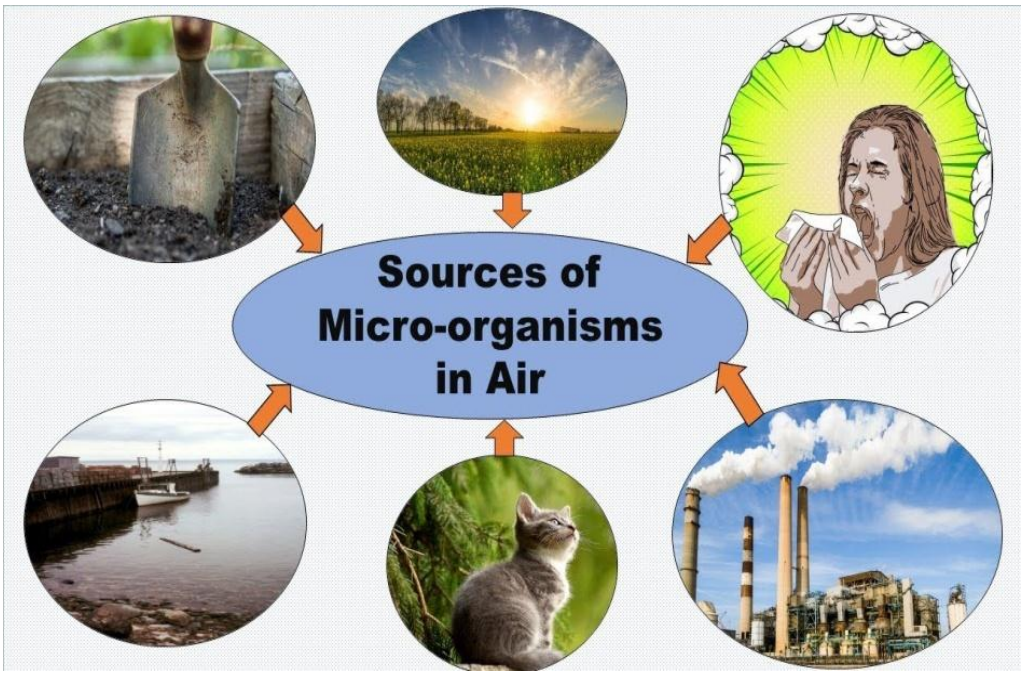
For other species in disease-associated biofilms and biofilms arising from eukaryotes,

Aeromicrobiology is the study of living microbes which are suspended in the air. These microbes are referred to as bioaerosols (Brandl et. al, 2008).



Though there are significantly less atmospheric microorganisms than there are in oceans and in soil, there is still a large enough number that they can affect the atmosphere (Amato, 2012). Once suspended in the air column, these microbes have the opportunity to travel long distances with the help of wind and precipitation, increasing the occurrence of widespread disease by these microorganisms. These aerosols are ecologically significant because they can be associated with disease in humans, animals and plants. Typically microbes will be suspended in clouds, where they are able to perform processes that alter the chemical composition of the cloud, and may even induce precipitation. Physical Environment: There are many factors within the physical environment that affect the launching, transport and deposition of bioaerosols. Particles which become suspended in the air column arise mainly from 74 terrestrial and aquatic environments and are typically launched by air turbulence (Pepper 2011). Winds are the primary means of transport for bioaerosols.

Bioaerosols can be deposited by a number of mechanisms, including gravity pulling them down, making contact with surfaces, or combining with rain which pulls the particles back down to earth's surface. Atmosphere Along with water droplets, dust particles and other matter, air contains microbes (Al-Dagal 1990). Microbes follow a particular pathway in which they are suspended into the atmosphere. First they are launched into the air. The source of the launching of airborne microbes stems from humans, animals and vegetation. (Al-Dagal 1990). then they are transported (by various methods including winds, machinery and people) and finally are deposited somewhere new. The atmosphere can have a variety of physical characteristics, and can be very extreme in terms of the relative humidity, temperature and radiation. These factors play a huge role in what kinds of microbes will survive in the atmosphere and how long they will stay alive (Pepper 2011). Clouds One area that bioaerosols can be found in is within clouds. Cloud water is a mixture of organic and inorganic compounds suspended within moisture (contribution of microbial activity yo clouds). The conditions in clouds are not conducive to much life, as microbes present there must withstand freezing temperatures, the threat of desiccation, and extreme UV rays. Clouds are also an acidic environment, with a pH ranging from 3 to 7. Nevertheless, there are extremophile microbes which can withstand all of these environmental pressures. Clouds serve as a transport for these microbes, dispersing them over long distances (Amato 2012)



Water microbiology refers to the study of the microorganisms that live in water, or which can be transported from one habitat to another by water. Water can support the growth of many types of microorganisms. This can be advantageous. For example, the chemical activities of certain strains of yeasts provide us with beer and bread. As well, the growth of some bacteria in contaminated water can help digest the poisons from the water. However, the presence of other disease causing microbes in water is unhealthy and even life threatening. For example, bacteria that live in the intestinal tracts of humans and other warm blooded animals, such as Escherichia coli, Salmonella, Shigella, and Vibrio, can contaminate water 78 if feces enters the water. Contamination of drinking water with a type of Escherichia coli known as O157:H7 can be fatal. The contamination of the municipal water supply of Walkerton, Ontario, Canada in the summer of 2000 by strain O157:H7 sickened 2,000 people and killed seven people. The intestinal tract of warm-blooded animals also contains viruses that can contaminate water and cause disease. Examples include rotavirus, enteroviruses, and coxsackievirus. Another group of microbes of concern in water microbiology are protozoa. The two protozoa of the most concern are Giardia and Cryptosporidium. They live normally in the intestinal tract of animals such as beaver and deer. Giardia and Cryptosporidium form dormant and hardy forms called cysts during their life cycles. The cyst forms are resistant to chlorine, which is the most popular form of drinking water disinfection, and can pass through the filters used in many water treatment plants. If ingested in drinking water they can cause debilitating and prolonged diarrhea in humans, and can be life threatening to those people with impaired immune systems. Cryptosporidium contamination of the drinking water of Milwaukee, Wisconsin with in 1993 sickened more than 400,000 people and killed 47 people. Illness caused by these protozoans are becoming more prevalent in the United States, as urban areas continue to expand into what was previously relatively undisturbed wilderness. Many microorganisms are found naturally in fresh and saltwater. These include bacteria, cyanobacteria, protozoa, algae, and tiny animals such as rotifers. These can be important in the food chain that forms the basis of life in the water. For example, the microbes called cyanobacteria can convert the energy of the sun into the energy it needs to live. The plentiful numbers of these organisms in turn are used as food for other life.

The algae that thrive in water is also an important food source for other forms of life. A variety of microorganisms live in fresh water. The region of a water body near the shoreline that is termed the littoral zone is well lighted, shallow, and warmer than other regions of the water. Photosynthetic algae and bacteria that use light as energy thrive in this zone. Further away from the shore is the limnitic zone, which can be colder and sunlight only in the upper 100 feet or so. Photosynthetic microbes also live here. As the water deepens, temperatures become colder and the oxygen concentration and light in the water decrease. Now, microbes that require oxygen do not thrive. Instead, purple and green sulfur bacteria, which can grow without oxygen, dominate. Finally, at the bottom of fresh waters (the benthic zone), few microbes survive. Bacteria that can survive in the absence of oxygen and sunlight, such as methane producing bacteria, thrive. Salt water presents a different environment to microorganisms. The higher salt concentration, higher pH, and lower nutrients, relative to freshwater, are lethal to many microorganisms. But, salt loving (halophilic) bacteria abound near the surface, and some bacteria that also live in freshwater are plentiful (i.e., Pseudomonas and Vibrio). Also, in 2001, researchers demonstrated that the ancient form of microbial life known as archaebacteria is one of the dominant forms of life in the ocean. The role of archaebacteria in the ocean food chain is not yet known, but must be of vital importance

Microorganisms exist at all depths in the ocean, even at the ocean floor, thousands of feet below the surface. Indeed, at locations such as hydrothermal vents, bacteria are the basis of the community of organisms that flourishes around the vents. Another microorganism found in saltwater are a type of algae known as dinoflagellelates. The rapid growth and multiplication of dinoflagellates can turn the water red. This “red tide” depletes the water of nutrients and oxygen, which can cause many fish to die. As well, humans can become ill by eating contaminated fish. Water can also be an ideal means of transporting microorganisms from one place to another. For example, the water that is carried in the hulls of ships to stabilize the vessels during their ocean voyages is now known to be a means of transporting microorganisms around the globe. One of these organisms, a bacterium called Vibrio cholerae, causes life threatening diarrhea in humans. Drinking water is usually treated to minimize the risk of microbial contamination. The importance of drinking water treatment has been known for centuries. For example, in pre-Christian times the storage of drinking water in jugs made of metal was practiced.

Now, the anti-bacterial effect of some metals is known. Similarly, the boiling of drinking water, as a means of protection of water has long been known. Chemicals such as chlorine or chlorine derivatives have been a popular means of killing bacteria such as Escherichia coli in water since the early decades of the twentieth century. Other bacteriakilling treatments that are increasingly becoming popular include the use of a gas called ozone and the disabling of the microbe's genetic material by the use of ultraviolet light. Microbes can also be physically excluded from the water by passing the water through a filter. Modern filters have holes in them that are so tiny that even particles as miniscule as viruses can be trapped. An important aspect of water microbiology, particularly for drinking water, is the testing of the water to ensure that it is safe to drink. Water quality testing can be done in several ways. One popular test measures the turbidity of the water. Turbidity gives an indication of the amount of suspended material in the water. Typically, if material such as soil is present in the water then microorganisms will also be present. The presence of particles even as small as bacteria and viruses can decrease the clarity of the water. Turbidity is a quick way of indicating if water quality is deteriorating, and so if action should be taken to correct the water problem. In many countries, water microbiology is also the subject of legislation. Regulations specify how often water sources are sampled, how the sampling is done, how the analysis will be performed, what microbes are detected, and the acceptable limits for the target microorganisms in the water sample. Testing for microbes that cause disease (i.e., Salmonella typhymurium and Vibrio cholerae) can be expensive and, if the bacteria are present in low numbers, they may escape detection. Instead, other more numerous bacteria provide an indication of fecal pollution of the water. Escherichia coli has been used as an indicator of fecal pollution for decades. The bacterium is present in the intestinal tract in huge numbers, and is more numerous than the disease-causing bacteria and viruses. The chances of detecting Escherichia coli are better than detecting the actual disease causing microorganisms. Escherichia coli also has the advantage of not being capable of growing and reproducing in the water (except in the warm and food-laden waters of tropical 80 countries). Thus, the presence of the bacterium in water is indicative of recent fecal pollution. Finally, Escherichia coli can be detected easily and inexpensively. Bacteria, viruses, and fungi are widely distributed throughout aquatic environments. They can be found in fresh water rivers, lakes, and streams, in the surface waters and sediments of the world's oceans, and even in hot springs. They have even been found supporting diverse communities at hydrothermal vents in the depths of the oceans.

Microorganisms living in these diverse environments must deal with a wide range of physical conditions, and each has specific adaptations to live in the particular place it calls home. For example, some have adapted to live in fresh waters with very low salinity, while others live in the saltiest parts of the ocean. Some must deal with the harsh cold of arctic waters, while those in hot springs are subjected to intense heat. In addition, aquatic microorganisms can be found living in environments where there are extremes in other physical parameters such as pressure, sunlight, organic substances, dissolved gases, and water clarity. Aquatic microorganisms obtain nutrition in a variety of ways. For example, some bacteria living near the surface of either fresh or marine waters, where there is often abundant sunlight, are able to produce their own food through the process of photosynthesis . Bacteria living at hydrothermal vents on the ocean floor where there is no sunlight can produce their own food through a process known as chemosynthesis , which depends on preformed organic carbon as an energy source. Many other microorganisms are not able to produce their own food. Rather, they obtain necessary nutrition from the breakdown of organic matter such as dead organisms. Aquatic microorganisms play a vital role in the cycling of nutrients within their environment, and thus are a crucial part of the food chain/web. Many microorganisms obtain their nutrition by breaking down organic matter in dead plants and animals. As a result of this process of decay, nutrients are released in a form usable by plants. These aquatic microorganisms are especially important in the cycling of the nutrients nitrogen, phosphorus, and carbon. Without this recycling, plants would have few, if any, organic nutrients to use for growth. In addition to breaking down organic matter and recycling it into a form of nutrients that plants can use, many of the microorganisms become food themselves. There are many types of animals that graze on bacteria and fungi. For example, some deposit-feeding marine worms ingest sediments and digest numerous bacteria and fungi found there, later expelling the indigestible sediments. Therefore, these microorganisms are intimate members of the food web in at least two ways. Humans have taken advantage of the role these microorganisms play in nutrient cycles. At sewage treatment plants, microscopic bacteria are cultured and then used to break down human wastes. However, in addition to the beneficial uses of some aquatic microorganisms, others may cause problems for people because they are pathogens, which can cause serious diseases. For example, viruses such as Salmonella typhi, S. paratyphi, and the Norwalk virus are found in water contaminated by sewage can cause illness.

Fecal coliform (E. coli) bacteria and Enterococcus bacteria are two types of microorganisms that are used to indicate the presence of disease causing microorganisms in aquatic environments.

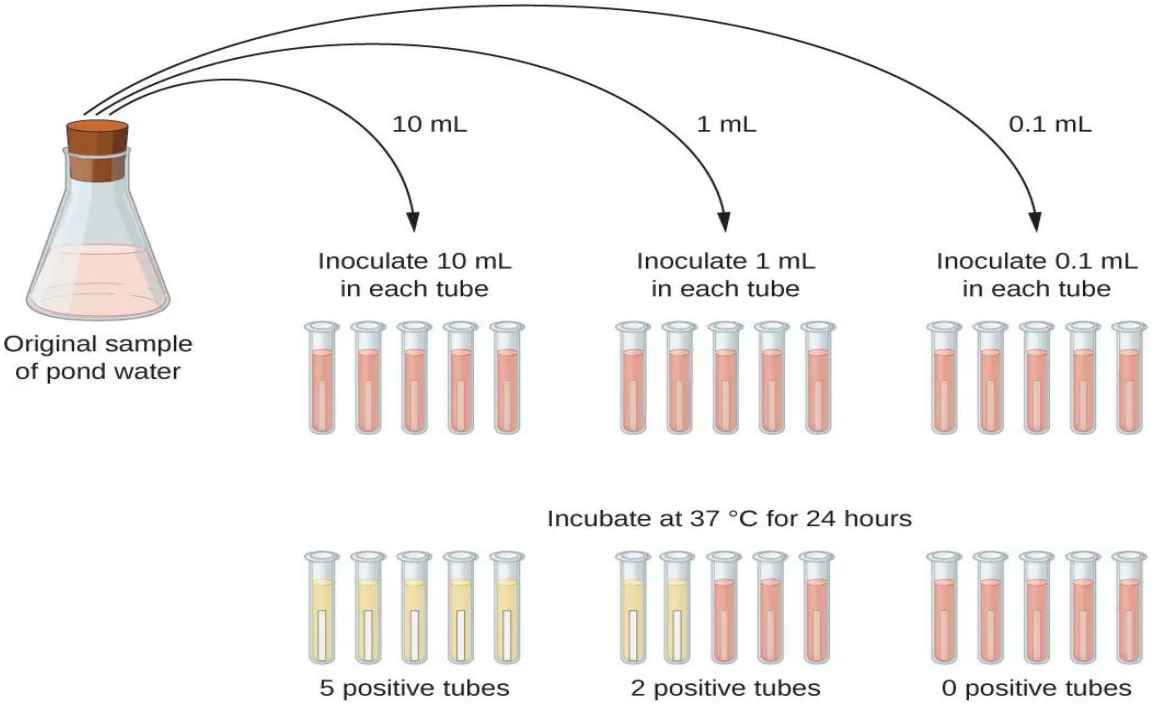
Multiple tube method- One of the oldest methods is called the multiple tube method. In this method a measured sub-sample (perhaps 10 ml) is diluted with 100 ml of sterile growth medium and an aliquot of 10 ml is then decanted into each of ten tubes. The remaining 10 ml is then diluted again and the process repeated. At the end of 5 dilutions this produces 50 tubes covering the dilution range of 1:10 through to 1:10000. The tubes are then incubated at a pre-set temperature for a specified time and at the end of the process the number of tubes with growth in is counted for each dilution. Statistical tables are then used to derive the concentration of organisms in the original sample. This method can be enhanced by using indicator medium which changes colour when acid forming species are present and by including a tiny inverted tube called a Durham tube in each sample tube. The Durham inverted tube catches any gas produced. The production of gas at 37 degrees Celsius is a strong indication of the presence of Escherichia coli.

ATP Testing- An ATP test is the process of rapidly measuring active microorganisms in water through detection adenosine triphosphate (ATP). ATP is a molecule found only in and around living cells, and as such it gives a direct measure of biological concentration and health. ATP is quantified by measuring the light produced through its reaction with the naturally occurring enzyme firefly luciferase using a luminometer. The amount of light produced is directly proportional to the amount of biological energy present in the sample. Second generation ATP tests are specifically designed for water, wastewater and industrial applications where, for the most part, samples contain a variety of components that can interfere with the ATP assay. Plate countThe plate count method relies on bacteria growing a colony on a nutrient medium so that the colony becomes visible to the naked eye and the number of colonies on a plate can be counted. To be effective, the dilution of the original sample must be arranged so that on average between 30 and 300 colonies of the target bacteria are grown. Fewer than 30 colonies makes the interpretation statistically unsound whilst greater than 300 colonies often results in overlapping colonies and imprecision in the count. To ensure that an appropriate number of colonies will be generated several dilutions are normally cultured. This approach is widely utilized for the evaluation of the effectiveness of water treatment by the inactivation of representative microbial contaminants such as E. coli following ASTM D5465.

The laboratory procedure involves making serial dilutions of the sample (1:10, 1:100, 1:1000, etc.) in sterile water and cultivating these on nutrient agar in a dish that is sealed and incubated. Typical media include plate count agar for a general count or MacConkey agar to count Gram-negative bacteria such as E. coli. Typically one set of plates is incubated at 22 °C and for 24 hours and a second set at 37 °C for 24 hours. The composition of the nutrient usually includes reagents that resist the growth of non-target organisms and make the target organism easily identified, often by a colour change in the medium. Some recent methods include a fluorescent agent so that counting of 83 the colonies can be automated. At the end of the incubation period the colonies are counted by eye, a procedure that takes a few moments and does not require a microscope as the colonies are typically a few millimetres across.

Membrane filtration- Most modern laboratories use a refinement of total plate count in which serial dilutions of the sample are vacuum filtered through purpose made membrane filters and these filters are themselves laid on nutrient medium within sealed plates. The methodology is otherwise similar to conventional total plate counts. Membranes have a printed millimetre grid printed on and can be reliably used to count the number of colonies under a binocular microscope.

Pour plate methodWhen the analysis is looking for bacterial species that grow poorly in air, the initial analysis is done by mixing serial dilutions of the sample in liquid nutrient agar which is then poured into bottles which are then sealed and laid on their sides to produce a sloping agar surface. Colonies that develop in the body of the medium can be counted by eye after incubation. The total number of colonies is referred to as the total viable count (TVC). The unit of measurement is cfu/ml (or colony forming units per millilitre) and relates to the original sample. Calculation of this is a multiple of the counted number of colonies multiplied by the dilution used.

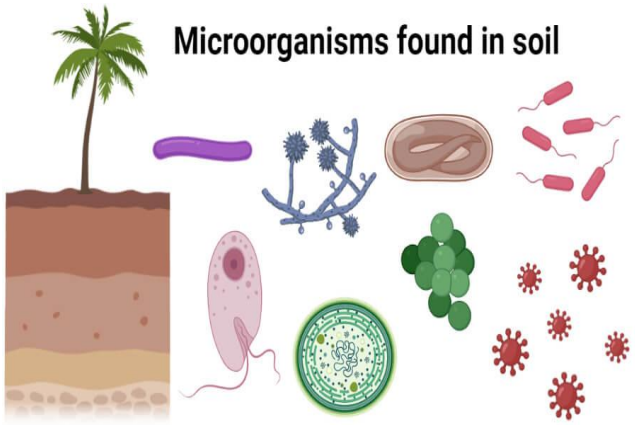


Most Probable Number (MPN) Test

Soil microbiology is the study of microorganisms in soil, their functions, and how they affect soil properties. It is believed that between two and four billion years ago, the first ancient bacteria and microorganisms came about on Earth's oceans. These bacteria could fix nitrogen, in time multiplied, and as a result released oxygen into the atmosphere. This led to more advanced microorganisms, which are important because they affect soil structure and fertility. Soil microorganisms can be classified as bacteria, actinomycetes, fungi, algae and protozoa. Each of these groups has characteristics that define them and their functions in soil. Up to 10 billion bacterial cells inhabit each gram of soil in and around plant roots, a region known as the rhizosphere. In 2011, a team detected more than 33,000 bacterial and archaeal species on sugar beet roots. The composition of the rhizobiome can change rapidly in response to changes in the surrounding environment. The microbial population of asoil will vary according to the amount of available waterand organic matter, and different organisms colonisedifferent strata in the soil.The organic content of a soil derives from the remainsof dead plants and animals. These are brokendown in the soil by a combination of invertebrates andmicroorganisms (mainly bacteria and fungi) known asthe decomposers. Their action results in the release ofsubstances that can be used by plants and by other microorganisms.

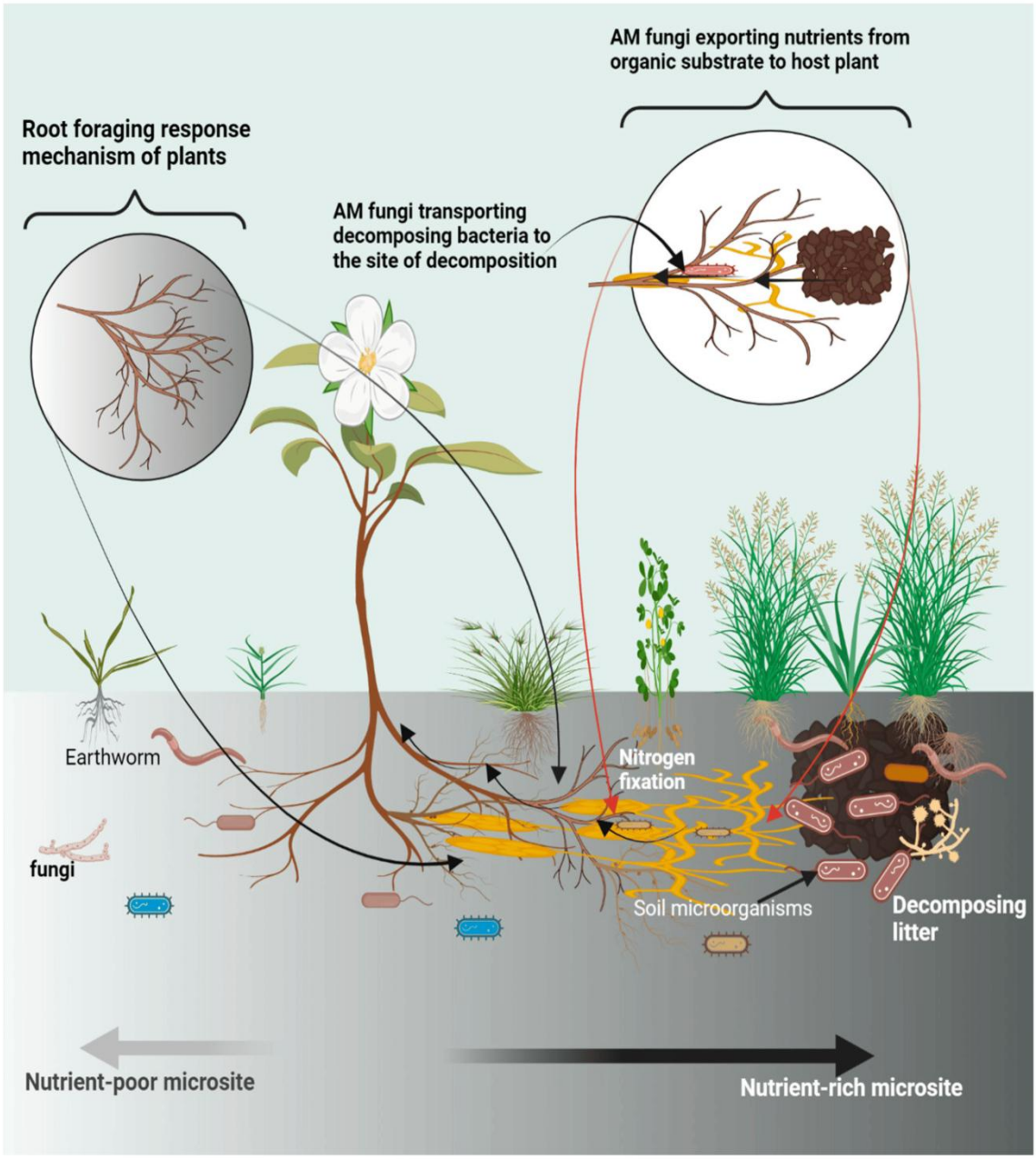
Much organic material is easily degraded,while the more resistant fraction is referred to as humus,and comprises lignin together with various othermacromolecules. The humus content of a soil, then, isa reflection of how favorable (or otherwise) conditionsare for its decomposition; the value usually falls between2 and 10 per cent by weight. The inorganic fraction 100 ofa soil derives from the weathering of minerals. Microorganisms may be present in soilsin huge numbers, mostly attached to soil particles. Their numbers vary according tothe availability of suitable nutrients. Bacteria (notably actinomycetes) form the largestfraction of the microbial population, together with much smaller numbers of fungi,algae and protozoans. Published values of bacterial numbers range from overestimates(those that do not distinguish between living and dead cells) and underestimates (thosethat depend on colony counts and therefore exclude those organisms we are not yetable to grow in the laboratory – 99 per cent according to some experts!). Suffice tosay that many millions (possibly billions) of bacteria may be present in a single gramof topsoil. In spite of being present in such enormous numbers, microorganisms onlyrepresent a minute percentage of the volume of most soils. Fungi, although present inmuch smaller numbers than bacteria, form a higher proportion of the soil biomass,due to their greater size. The majority of soil microorganisms are aerobic heterotrophs,involved in the decomposition of organic substrates; thus, microbial numbers diminishgreatly the further down into the soil we go, away from organic matter and oxygen.The proportion of anaerobes increases with depth, but unless the soil is waterlogged,they are unlikely to predominate. Other factors affecting microbial distribution include pH, temperature, and moisture.Broadly speaking, neutral conditions favour bacteria, while fungi flourish in mildlyacidic conditions (down to about pH4), although extremophiles survive well outsidethese limits. Actinomycetes favour slightly alkaline conditions. Bacterial forms occurringcommonly in soils include Pseudomonas, Bacillus, Clostridium, Nitrobacter and the nitrogen-fixing Rhizobium and Azotobacter, as well as cyanobacteria such as Nostocand Anabaena. Commonly found actinomycetes include Streptomyces and Nocardia. As we have noted elsewhere, actinomycetes are notable for their secretion of antimicrobial compounds into their surroundings. This provides an example of how the presence of one type of microorganism in a soil population can influence the growth of others, forming a dynamic, interactive ecosystem. In addition, bacteria may serve as prey for predatory protozoans, and secondary colonisers may depend on a supply of nutrients from, for example, cellulose degraders.

Important fungal genera common in soil include the familiar Penicillium and Aspergillus; these not only recycle nutrients by breaking down organic material, but also contribute to the fabric of the soil, by binding together microscopic soil particles. Soil protozoans are mostly predators that ingest bacteria or protists such as yeasts or unicellular algae. All the major forms of protozoans may be present (flagellates, ciliates and amoebas), moving around the water-lined spaces between soil particles. Algae are of course phototrophic, and are therefore to be found mostly near the soil surface, although it will be recalled from that some forms are capable of heterotrophic growth, and may thus survive further down. The surface of soil particles is a good natural habitat for the development of biofilms, complex structures comprising microbial cells held together in a polysaccharide matrix.The microorganisms themselves produce the polysaccharide, which also allows the passageof nutrients from the environment. Biofilms can form on almost any surface, and areoften to be found in rapidly flowing waters. Biofilms may be beneficial or harmful (e.g. infections resulting from growth in catheters)to humans. 101 Although we have emphasized the importance of organic matter in soil ecosystems,microorganisms may also be found growing on or even within rocks. The growth of suchorganisms, together with the action of wind and rainfall, contribute to the weatheringof rocks. Terrestrial Microbiology: The Composition Descriptions such as “soiled” or “dirty” may suggest to some that soil is an undesirable, possibly harmful substance; or its appearance mightsuggest a somewhat homogeneous, inert substance. At the microscopiclevel, however, soil is a dynamic ecosystem that supports complexinteractions between numerous geologic, chemical, and biologicalfactors. This rich region, part of the lithosphere, teems with microbes,serves a dynamic role in biogeochemical cycles, and is an importantrepository for organic detritus and dead terrestrial organisms. The abiotic portion of soil is a composite of mineral particles,water, and atmospheric gas. The development of soil begins whengeologic sediments are mechanically disturbed and exposed toweather and microbial action. Rock decomposition releases various-size particles rangingfrom rocks, pebbles, and sand grains to microscopic morsels that liein a loose aggregate.



The porous structure of soil creates various-size pockets or spaces that provide numerous microhabitats.Some spaces trap moisture and form a liquid phase in whichmineral ions and other nutrients are dissolved. Other spaces trap airthat will provide gases to soil microbes, plants, and animals. Becauseboth water and air compete for these pockets, the water content ofsoil is directly related to its oxygen content. Water-saturated soilscontain less oxygen, and dry soils have more. Gas tensions in soil canalso vary vertically. In general, the concentration of O 2 decreases andthat of CO 2 increases with the depth of soil. Aerobic and facultativeorganisms tend to occupy looser, drier soils, whereas anaerobeswould adapt to waterlogged, poorly aerated soils. Within the superstructure of the soil are varying amounts ofhumus, * the slowly decaying organic litter from plant and animaltissues. This soft, crumbly mixture holds water like a sponge. It isalso an important habitat for microbes that decompose the complexlitter and recycle nutrients.

- **deep sea**: The deeper part of the sea or ocean in which no light penetrates.
- **piezophile**: A piezophile (also called a barophile) is an organism which thrives at high pressures, such as deep sea bacteria or archaea.
- **chemosynthesis**: The production of carbohydrates and other compounds from simple compounds such as carbon dioxide, using the oxidation of chemical nutrients as a source of energy rather than sunlight; it is limited to certain bacteria and fungi.



Potential Roles of Soil Microorganisms in Regulating the Effect of Soil Nutrient Heterogeneity on Plant Performance

Biochemical activity

Most soil enzymes are produced by bacteria, fungi and plant roots. Their biochemical activity is a factor in both stabilization and degradation of soil structure. Enzyme activity is higher in plots that are fertilized with manure as compared to inorganic fertilizers. The microflora of the rhizosphere may increase activity of enzymes there.

Applications

Agriculture

Microbes can make nutrients and minerals in the soil available to plants, produce hormones that spur growth, stimulate the plant immune system and trigger or dampen stress responses. In general a more diverse soil microbiome results in fewer plant diseases and higher yield.

Farming can destroy soil's rhiziobiome (microbial ecosystem) by using soil amendments such as fertilizer and pesticide without compensating for their effects. By contrast, healthy soil can increase fertility in multiple ways, including supplying nutrients such as nitrogen and protecting against pests and disease, while reducing the need for water and other inputs. Some approaches may even allow agriculture in soils that were never considered viable.

The group of bacteria called rhizobia live inside the roots of legumes and fix nitrogen from the air into a biologically useful form.

Mycorrhizae or root fungi form a dense network of thin filaments that reach far into the soil, acting as extensions of the plant roots they live on or in. These fungi facilitate the uptake of water and a wide range of nutrients.

Up to 30% of the carbon fixed by plants is excreted from the roots as so-called exudates—including sugars, amino acids, flavonoids, aliphatic acids, and fatty acids—that attract and feed beneficial microbial species while repelling and killing harmful ones.

Commercial activity

Further information: Microbial inoculant

Almost all registered microbes are biopesticides, producing some $1 billion annually, less than 1% of the chemical amendment market, estimated at $110 billion. Some microbes have been marketed for decades, such as Trichoderma fungi that suppress other, pathogenic fungi, and the caterpillar killer Bacillus thuringiensis. Serenade is a biopesticide containing a Bacillus subtilis strain that has antifungal and antibacterial properties and promotes plant growth. It can be applied in a liquid form on plants and to soil to fight a range of pathogens. It has found acceptance in both conventional and organic agriculture.

Agrochemical companies such as Bayer have begun investing in the technology. In 2012, Bayer bought AgraQuest for $425 million. Its €10 million annual research budget funds field-tests of dozens of new fungi and bacteria to replace chemical pesticides or to serve as biostimulants to promote crop health and growth. Novozymes, a company developing microbial fertilizers and pesticides, forged an alliance with Monsanto. Novozymes invested in a biofertilizer containing the soil fungus Penicillium bilaiae and a bioinsecticide that contains the fungus Metarhizium anisopliae. In 2014 Syngenta and BASF acquired companies developing microbial products, as did Dupont in 2015.

A 2007 study showed that a complex symbiosis with fungi and viruses makes it possible for a grass called Dichanthelium lanuginosum to thrive in geothermal soils in Yellowstone National Park, where temperatures reach 60 °C (140 °F). Introduced in the US market in 2014 for corn and rice, they trigger an adaptive stress response.

In both the US and Europe, companies have to provide regulatory authorities with evidence that both the individual strains and the product as a whole are safe, leading many existing products to label themselves "biostimulants" instead of "biopesticides".

When selecting a bacterium for disease control its other effects must also be considered. Some suppressive bacteria perform the opposite of nitrogen fixation (see § Nitrogen fixation above), making nitrogen unavailable. Stevens et al 1998 find bacterial denitrification and dissimilatory nitrate reduction to ammonium to especially occur at high pH.

Unhelpful microbes

A funguslike unicellular organism named Phytophthora infestans, responsible for potato blight and other crop diseases, has caused famines throughout history. Other fungi and bacteria cause the decay of roots and leaves.

Many strains that seemed promising in the lab often failed to prove effective in the field, because of soil, climate and ecosystem effects, leading companies to skip the lab phase and emphasize field tests.

Fade

Populations of beneficial microbes can diminish over time. Serenade stimulates a high initial B. subtilis density, but levels decrease because the bacteria lacks a defensible niche. One way to compensate is to use multiple collaborating strains.

Fertilizers deplete soil of organic matter and trace elements, cause salination and suppress mycorrhizae; they can also turn symbiotic bacteria into competitors.

Pilot project

A pilot project in Europe used a plow to slightly loosen and ridge the soil. They planted oats and vetch, which attracts nitrogen-fixing bacteria. They planted small olive trees to boost microbial diversity. They split an unirrigated 100-hectare field into three zones, one treated with chemical fertilizer and pesticides; and the other two with different amounts of an organic biofertilizer, consisting of fermented grape leftovers and a variety of bacteria and fungi, along with four types of mycorrhiza spores.

The crops that had received the most organic fertilizer had reached nearly twice the height of those in zone A and were inches taller than zone C. The yield of that section equaled that of irrigated crops, whereas the yield of the conventional technique was negligible. The mycorrhiza had penetrated the rock by excreting acids, allowing plant roots to reach almost 2 meters into the rocky soil and reach groundwater.

Deep sea ecosystem and barotolerant and barophilic bacteria; Deep sea communities currently remain largely unexplored, due the technological and logististical challenges, and the expense involved in visiting these remote biomes. Because of the unique challenges (particularly the high barometric pressure, extremes of temperature, and absence of light), it was long believed that little life existed in this hostile environment. Since the 19^{th} century however, research has demonstrated that significant biodiversity exists in the deep sea.

The three main sources of energy and nutrients for deep sea communities are marine snow, whale falls, and chemosynthesis at hydrothermal vents and cold seeps.

Zones of the deep sea include the mesopelagic zone, the bathyal zone, the abyssal zone, and the hadal zone.

A piezophile, also called a barophile, is an organism which thrives at high pressures, such as deep sea bacteria or archaea. They are generally found on ocean floors, where pressure often exceeds 380 atm (38 MPa). Some have been found at the bottom of the Pacific Ocean where the maximum pressure is roughly 117 MPa. The high pressures experienced by these organisms can cause the normally fluid cell membrane to become waxy and relatively impermeable to nutrients.

These organisms have adapted in novel ways to become tolerant of these pressures in order to colonize deep sea habitats. One example, xenophyophores, have been found in the deepest ocean trench, 6.6 miles (10,541 meters) below the surface.

Barotolerant bacteria are able to survive at high pressures, but can exist in less extreme environments as well. Obligate barophiles cannot survive outside of such environments. For example, the Halomonas species Halomonas salaria requires a pressure of 1000 atm (100 MPa) and a temperature of three degrees Celsius. Most piezophiles grow in darkness and are usually very UV-sensitive; they lack many mechanisms of DNA repair.

MECHANISMS OF ADAPTATION

The mechanisms by which marine bacteria adapt to high pressures are very inadequately understood, but pressureregulated gene expression and its relationship to barophily and barotolerance are gradually being determined. Pressureregulated genes are believed to aid pressure acclimatization in marine bacteria that are exposed to large vertical changes in the water column, but they are also found in bacteria that are not subject to pressure changes as a result of overlapping effects of pressure and other environmental stresses (Bartlett et al, 1995). To date most work on deep-sea barophilic bacteria has concerned taxon within the Proteobacteria: Colwellia, Moritella, Photobacterium, and Shewanella and an unidentified genus (DeLong et al, 1997). Some of these bacteria are extremely barophilic, such as the newly described species Moritella yayanosii isolated from the Challenger Deep of the Mariana Trench, which grows at 60 to 100 MPa and has an optimum of 70 MPa (Nogi and Kato, 1999).

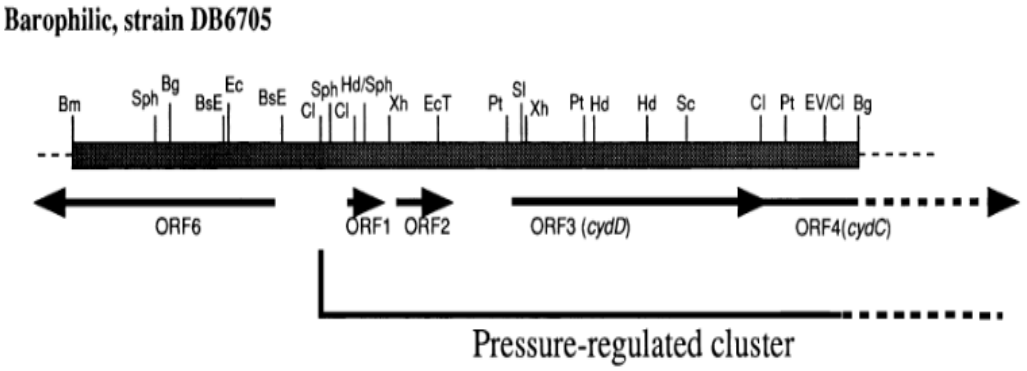


Fig. 2. Restriction map of the DNA fragment containing the two pressure-regulated operons and flanking open reading frames (*ORF*s) from the barophilic strain DB6705, and the structure of the genes. *Arrows* indicate open reading frames. Restriction endonucleases are indicated as follows: Bg, *BglII*; Bm, *Bam*HI; BsE, *Bst*EII; Cl, *Cla*I; Ec, *Eco*RI; EcT, *Eco*T22; EV, *Eco*RV; Hd, *Hin*dIII; Pt, *Pst*I; Sc, *Sac*I; Sl, *Sal*I; Sph, *Sph*I; and Xh, *Xho*I. The accession number of this DNA sequence from strain DB6705 is D88688 in the DDBJ, EMBL, and GenBank DNA databases

Promising progress has been made on molecular mechanism of deep-sea Photobacterium and Shewanella strains. Reverse-pressure regulation of outer membrane proteins has been shown in the moderate barophile Photobacterium profundum SS9. A 10- to 100-fold increase in the expression of the OmpH protein occurs at high pressures (28 MPa), while at 0.1 MPa the OmpL protein is produced in greatest quantity. Furthermore, a third pressure-regulated protein, OmpI, is expressed at 40 MPa. The OmpH protein is believed to be a relatively nonspecific porin (Welch and Bartlett, 1998) that may facilitate nutrient uptake under increasingly oligotrophic conditions of the deep-sea. More recently it has been demonstrated that RecD function is required for high-pressure growth and maintenance of plasmid stability in P. profundum SS9 (Bidle and Bartlett, 1999). The Japan Marine Science and Technology Center (JAMSTEC) group, whose main focus has been on deep-sea Shewanella strains, has distinguished a "barophilic branch" of Shewanella benthica strains from moderately barophilic and barotolerant strains (Li et al, 1998). Kato et al, (1995) reported that a pressure-regulated operon unidentified ORFs (ORF1 and ORF2) of the barophilic S. benthica to have a sequence similar to that of the OmpH promoter of P. profundum. In addition, a second pressureregulated operon (ORF3 and ORF4) is located downstream from the first operon; ORF3 encodes the CydD protein which is required for the assembly of the cytochrome bd complex (Kato et al, 1996). A truncated respiratory chain has been proposed for S. benthica at high pressure in which quinol oxidase acts as the terminal oxidase (Kato, 1999).

BIOTECHNOLOGICAL POTENTIALS OF DEEP-SEA BACTERIA

Several world wide oceanographic institutions have carried out exploration of deep-sea microorganisms, such as JAMSTEC Japan, Monterey Bay Aquarium Institute (MBARI), Woodshole Oceanographic Institution (WHOI), Scripps Institute of Oceanography (USA) and Ifremer of France. So far, from the cold deep-sea environments there have come new findings of unexpected microbial diversity and the promise of industrially useful enzymes or secondary metabolites. New classes of predictive models are emerging to guide future exploration of microbial diversity in the deep ocean. It is expected that still quite a few parts of culturable psy-chrophilic and barophilic microorganisms are explored in the deepsea environments. Thus, the discoveries of novel deep-sea bacteria possibly become sources of novel natural products, including lipids, pigments, pharmaceuticals and low temperature adapted enzymes.

Microbial communities that thrive in spite of pronounced biotic pressure as it is among deep-sea bacteria, can come to some degree be expected to contain metabolites that are also of interest bioactive prospector searching the oceans. It is then reasonable for marine microbiologists and biotechnologists to look into marine environment, and the deep-sea in particular as a source of novel microorganisms and exploitable properties. If deep-sea bacteria are indeed the producers of bioactive metabolites of interest, transfer of gene clusters responsible for the biosynthesis of the respective natural products to a vector suitable for large-scale fermentation could perhaps provide alternative strategy thereby avoiding the foreseeable difficulties in culturing bacteria. Low temperature adapted bacteria have been known to produce enzymes with special interest such as protease, lypase, galactosydase, pectinase, catalase and glucose oxidase (Ohgiya et al, 1999), in addition to another potential, new pigments for food additives. Lipid membrane of barophilic bacteria contains almost 70% of poly unsaturated fatty acids (PUFA) which are very useful to decrease the chollesterol, prevent cardiovascular disease, and reduce the risk of cancers (Kelly, 1991). PUFA are also needed for normal embrionic development, in particular for optical and neural (Craigschmitdt et al, 1996; Farkas et al, 1996; Linko and Hiyakawa, 1996). PUFA from marine bacteria also serve as the alternative sources in aquaculture field (Nichols et al, 1996). A work by Kojima et al (1998) was carried out as an effort to find alternative sources for synthetic surfactants which are widely used by industries. These surfactants in a large amount could affect the quality of ecosystems. Studies dedicated to obtaining sources of environmentally friendly and degradable surfactants were conducted in the area of Japan, Palau and Mariana Trenches.

Microbial Leaching Process

Bacteria perform the key reaction of regenerating the major ore oxidizer which in most cases is ferric iron as well as further ore oxidation. The reaction is performed at the bacterial cell membrane. In the process, free electrons are generated and used for the reduction of oxygen to water which produces energy in the bacterial cell.

Ores, like pyrite (FeS_2), are first oxidized by ferric iron (Fe^{3+}) to thiosulfate ($S_2O_3^{2-}$) in the absence of bacteria.

In the first step, disulfide is spontaneously oxidized to thiosulfate by ferric iron (Fe^{3+}), which in turn is reduced to give ferrous iron (Fe^{2+}):

(1)
FeS2+6Fe3++3H2O⟶7Fe2++S2O2−3+6H+spontaneousFeS2+6Fe3++3H2O⟶7Fe2++S2O32−+6H+spontaneous

Bacteria are added in the second step and recover Fe^{3+} from ferrous iron (Fe^{2+}) which is then reused in the first step of leaching:

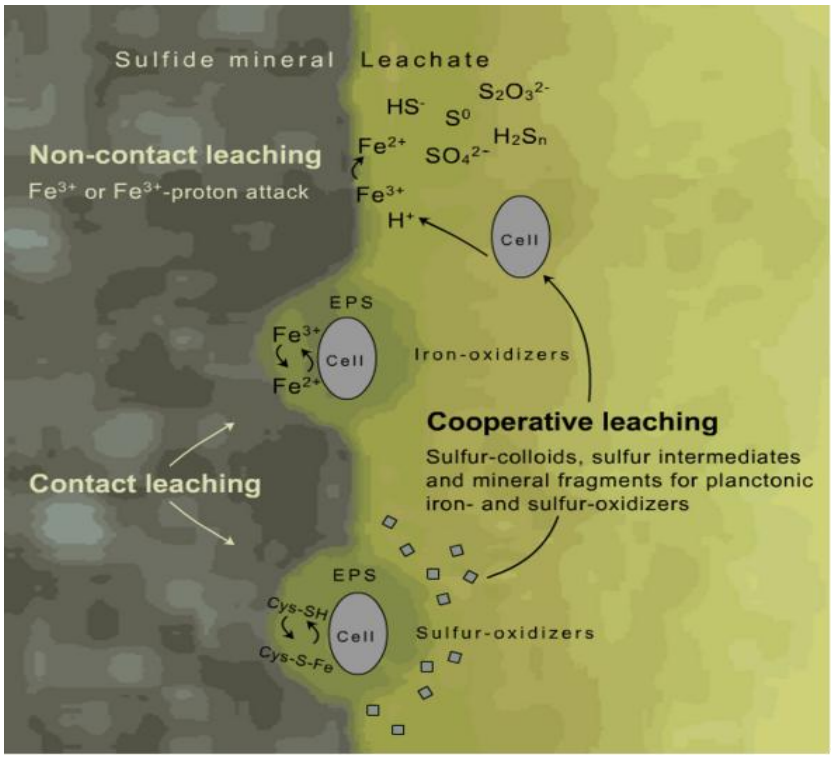


Figure: **Sulfide mineral bacterial leaching**: Bacterial cells oxidizing the ferrous iron back to ferric iron while using slightly different contact mechanisms with the metal.

(2) $4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O$(iron oxidizers)$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O$(iron oxidizers)

Thiosulfate is also oxidized by bacteria to give sulfate:

(3) $S_2O^{2-}_3 + 2O_2 + H_2O \longrightarrow 2SO^{2-}_4 + 2H^+$(sulfur oxidizers)$S_2O_3^{2-} + 2O_2 + H_2O \longrightarrow 2SO_4^{2-} + 2H^+$(sulfur oxidizers)

The ferric iron produced in reaction (2) oxidized more sulfide as in reaction (1), closing the cycle and given the net reaction:

(4) $2FeS_2 + 7O_2 + 2H_2O \longrightarrow 2Fe^{2+} + 4SO^{2-}_4 + 4H^+ 2FeS_2 + 7O_2 + 2H_2O \longrightarrow 2Fe^{2+} + 4SO_4^{2-} + 4H^+$

The net products of the reaction are soluble ferrous sulfate and sulfuric acid.

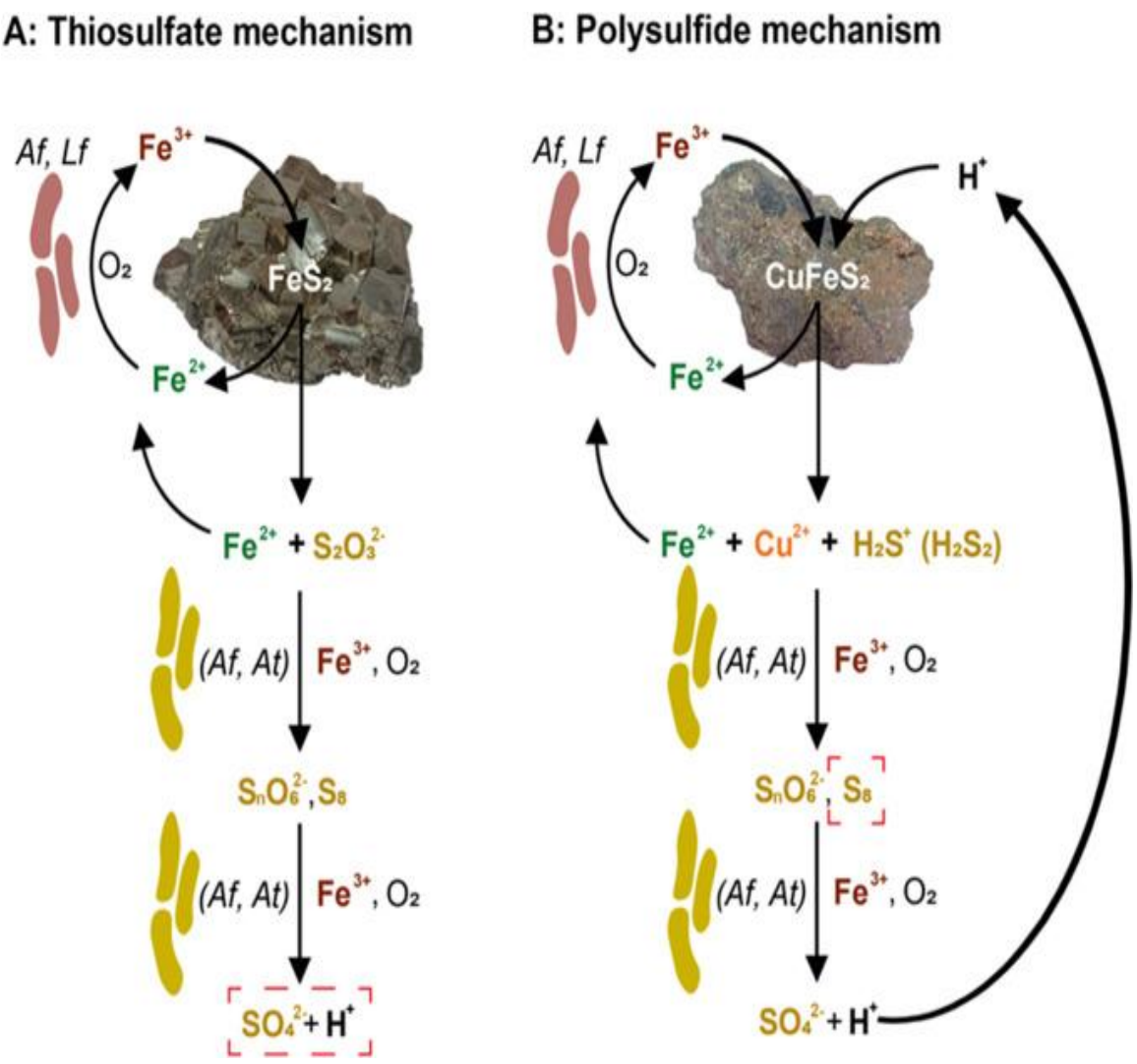
The microbial oxidation process occurs at the cell membrane of the bacteria. The electrons pass into the cells and are used in biochemical processes to produce energy for the bacteria while reducing oxygen to water. The critical reaction is the oxidation of sulfide by ferric iron. The main role of the bacterial step is the regeneration of this reactant.

Copper leaching has a very similar mechanism.

Microorganisms Capable of Ore Leaching

Bioleaching reactions industrially are performed by many bacterial species that can oxidize ferrous iron and sulfur. An example of such species is *Acidithiobacillus ferroxidans*. Some fungi species (*Aspergillus niger* and *Penicillium simplicissimum*) have also been shown to have the ability to dissolute heavy metals. When fungi are used, the leaching mechanism is different. The fungi use the acids that they produce in their metabolic reactions to dissolve the metal.

In general, bioleaching is cleaner and safer for the environment than chemical processing. However environmental pollution with toxic products, like sulfuric acid from the pyrite leaching, and heavy metals is still possible. Another drawback of microbial leaching is the slow rate at which microbes work.



Schematic comparison of the thiosulfate (A) and polysulfide (B) pathways in (bio)leaching of metal sulfides (from Schippers and Sand (1999), modified). Iron (III) ions attack metal sulfides (MS) by electron extraction and are thereby reduced to iron(II) ions. As a result, the metal sulfide mineral releases metal cations ($M.^{2+)}$ and water-soluble intermediary sulfur compounds. Iron(II)-oxidizing bacteria such as At. ferrooxidans (Af) and L. ferrooxidans (Lf) catalyze the re-oxidation of iron(II) to iron(III) ions in acidic solutions. In the case of acid-soluble metal sulfides (B), an additional attack is performed by protons, which can bind valence band electrons of these metal sulfides. The liberated sulfur compounds are oxidized abiotically and by sulfur compound-oxidizing bacteria such as At. ferrooxidans and At. thiooxidans (At). In the case of mainly abiotic reactions, the contribution of sulfur compound oxidizers is indicated in brackets. The main electron acceptors of oxidation reactions other than the initial iron(III) ion attack on the metal sulfide are given to the right of the arrows. The main reaction products that accumulate in the absence of sulfur compound oxidizers are boxed, i.e., sulfuric acid in (A) and elemental sulfur in (B). The equations given are not stoichiometric.

Biogeochemical cycle: Microbial Role in Biogeochemical Cycling

Nutrients move through the ecosystem in biogeochemical cycles. A biogeochemical cycle is a pathway by which a chemical element (such as carbon or nitrogen) circulates through the biotic (living) and the abiotic (non-living) factors of an ecosystem. The elements that move through the factors of an ecosystem are not lost but are instead recycled or accumulated in places called reservoirs (or "sinks") where they can be held for a long period of time. Elements, chemical compounds, and other forms of matter are passed from one organism to another and from one part of the biosphere to another through these biogeochemical cycles.

Ecosystems have many biogeochemical cycles operating as a part of the system. A good example of a molecule that is cycled within an ecosystem is water, which is always recycled through the water cycle. Water undergoes evaporation, condensation, and then falls back to Earth as rain (or other forms of precipitation). This typifies the cycling that is observed for all of the principal elements of life.

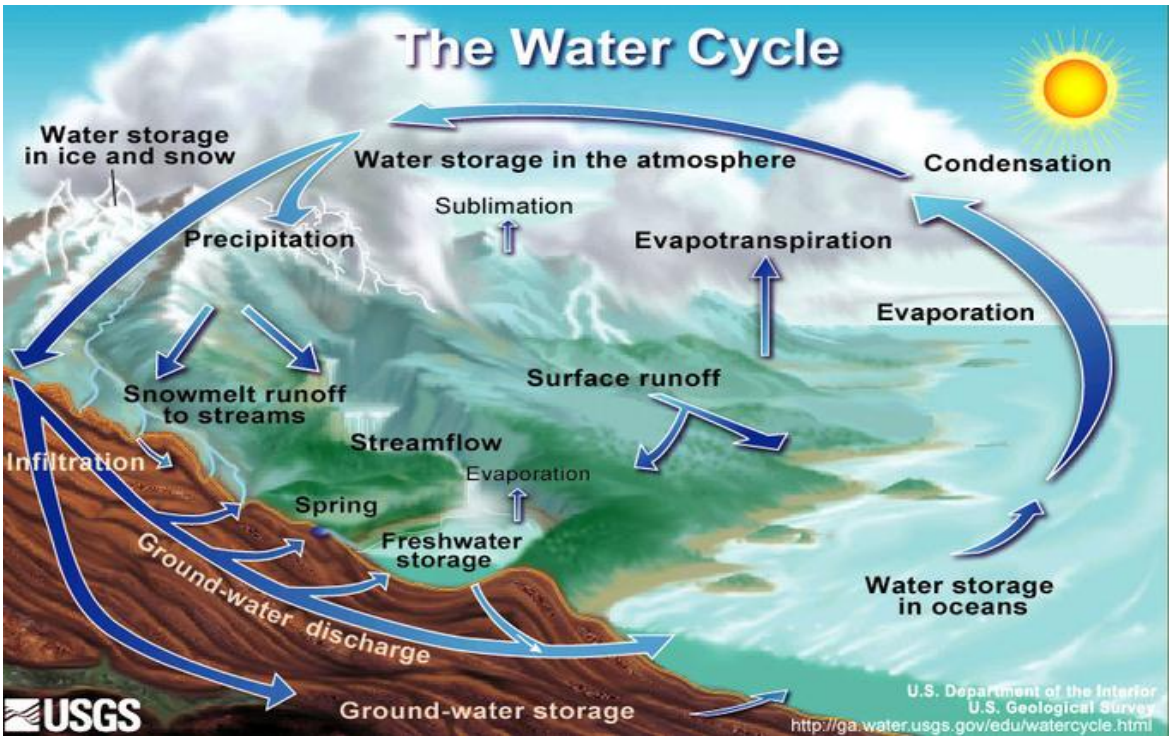
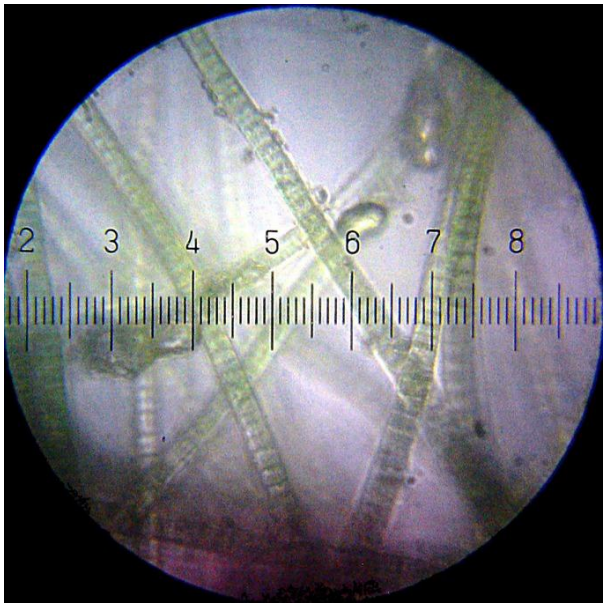


Figure: **The Water Cycle**: Water is recycled in an ecosystem through the water cycle.

Although biogeochemical cycles in a given ecosystem are coordinated by the full complement of living organisms and abiotic factors that make up that system, microorganisms play a primary role in regulating biogeochemical systems in virtually all of our planet's environments.

This includes extreme environments such as acid lakes and hydrothermal vents, and even includes living systems such as the human gut. The key collective metabolic processes of microbes (including nitrogen fixation, carbon fixation, methane metabolism, and sulfur metabolism) effectively control global biogeochemical cycling. Incredibly, production by microbes is so immense that global biogeochemistry would likely not change even if eukaryotic life were totally absent! Microbes comprise the backbone of every ecological system, particularly those in which there is no light (i.e. systems in which energy cannot be collected through photosynthesis).

Two key examples of critical biogeochemical processes carried out by microorganisms are discussed below.



The Carbon Cycle

Figure: **Cyanobacteria**: Cyanobacteria, also known as blue-green bacteria, blue-green algae, and Cyanophyta, is a phylum of bacteria that obtain their energy through photosynthesis

Carbon is critical for life because it is the essential building block of all organic compounds. Plants and animals utilize carbon to produce carbohydrates, fats, and proteins, which can then be used to build their internal structures or to obtain energy.

Carbon in the form of carbon dioxide (CO_2) is readily obtained from the atmosphere, but before it can be incorporated into living organisms it must be transformed into a usable organic form. The transformative process by which carbon dioxide is taken up from the atmospheric reservoir and "fixed" into organic substances is called carbon fixation. Perhaps the best known example of carbon fixation is photosynthesis, a process by which energy derived from sunlight is harnessed to form organic compounds. Photosynthesis depends on the activity of microorganisms such as cyanobacteria; indeed, the fact that there is oxygen in the Earth's atmosphere at all is a consequence of the photosynthetic activity of ancient microbes.

The Nitrogen Cycle Nitrogen is essential for all forms of life because it is required

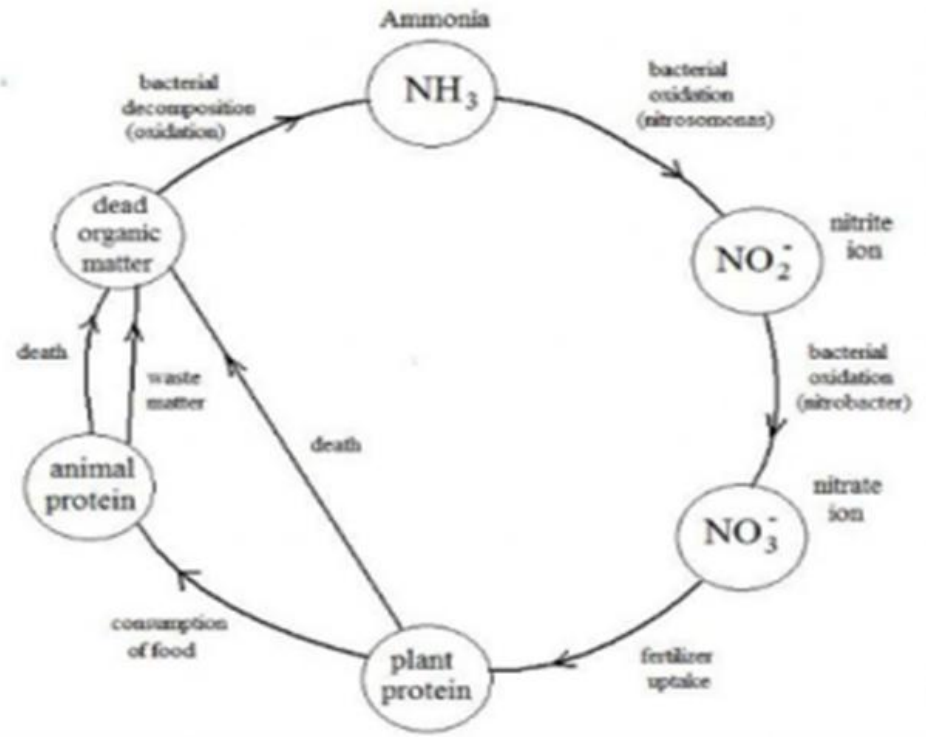


Figure: The Role of Microbes in the Nitrogen Cycle**:**

The processing of nitrogen into a biologically useful form requires the activity of microorganisms.

For synthesis of the basic building blocks of life (e.g., DNA, RNA, and amino acids). The Earth's atmosphere is primarily composed of nitrogen, but atmospheric nitrogen (N_2) is relatively unusable for biological organisms. Consequently, chemical processing of nitrogen (or nitrogen fixation) is necessary to convert gaseous nitrogen into forms that living organisms can use. Almost all of the nitrogen fixation that occurs on the planet is carried out by bacteria that have the enzyme nitrogenase, which combines N_2 with hydrogen to produce a useful form of nitrogen (such as ammonia). Thus, microorganisms are absolutely essential for plant and animal life forms, which cannot fix nitrogen on their own.

Microbial ecology (or **environmental microbiology**) is the ecology of microorganisms: their relationship with one another and with their environment. It concerns the three major domains of life—Eukaryota, Archaea, and Bacteria—as well as viruses.

Microorganisms, by their omnipresence, impact the entire biosphere. Microbial life plays a primary role in regulating biogeochemical systems in virtually all of our planet's environments, including some of the most extreme, from frozen environments and acidic lakes, to hydrothermal vents at the bottom of deepest oceans, and some of the most familiar, such as the human small intestine, nose, and mouth. As a consequence of the quantitative magnitude of microbial life (calculated as 5.0×10^{30} cells; eight orders of magnitude greater than the number of stars in the observable universe) microbes, by virtue of their biomass alone, constitute a significant carbon sink. Aside from carbon fixation, microorganisms' key collective metabolic processes (including nitrogen fixation, methane metabolism, and sulphur metabolism) control global biogeochemical cycling. The immensity of microorganisms' production is such that, even in the total absence of eukaryotic life, these processes would likely continue unchanged.

While microbes have been studied since the seventeenth century, this research was from a primarily physiological perspective rather than an ecological one. For instance, Louis Pasteur and his disciples were interested in the problem of microbial distribution both on land and in the ocean. Martinus Beijerinck invented the enrichment culture, a fundamental method of studying microbes from the environment. He is often incorrectly credited with framing the microbial biogeographic idea that "everything is everywhere, but, the environment selects", which was stated by Lourens Baas Becking. Sergei Winogradsky was one of the first researchers to attempt to understand microorganisms outside of the medical context—making him among the first students of microbial ecology and environmental microbiology—discovering chemosynthesis, and developing the Winogradsky column in the process.:

Beijerinck and Windogradsky, however, were focused on the physiology of microorganisms, not the microbial habitat or their ecological interactions. Modern microbial ecology was launched by Robert Hungate and coworkers, who investigated the rumen ecosystem. The study of the rumen required Hungate to develop techniques for culturing anaerobic microbes, and he also pioneered a quantitative approach to the study of microbes and their ecological activities that differentiated the relative contributions of species and catabolic pathways.

Progress in microbial ecology has been tied to the development of new technologies. The measurement of biogeochemical process rates in nature was driven by the availability of radioisotopes beginning in the 1950s. For example, $14CO_2$ allowed analysis of rates of photosynthesis in the ocean (ref).

Another significant breakthrough came in the 1980s, when microelectrodes sensitive to chemical species like O_2 were developed. These electrodes have a spatial resolution of 50–100 μm, and have allowed analysis of spatial and temporal biogeochemical dynamics in microbial mats and sediments.

Although measuring biogeochemical process rates could analyse what processes were occurring, they were incomplete because they provided no information on which specific microbes were responsible. It was long known that 'classical' cultivation techniques recovered less than 1% of the microbes from a natural habitat. However, beginning in the 1990s, a set of cultivation-independent techniques has evolved to determine the relative abundance of microbes in a habitat. Carl Woese first demonstrated that the sequence of the 16S ribosomal RNA molecule could be used to analyse phylogenetic relationships. Norm Pace took this seminal idea and applied it to analysfe 'who's there' in natural environments. The procedure involves (a) isolation of nucleic acids directly from a natural environment, (b) PCR amplification of small subunit rRNA gene sequences, (c) sequencing the amplicons, and (d) comparison of those sequences to a database of sequences from pure cultures and environmental DNA. This has provided tremendous insights into the diversity present within microbial habitats. However, it does not resolve how to link specific microbes to their biogeochemical role. Metagenomics, the sequencing of total DNA recovered from an environment, can provide insights into biogeochemical potential, whereas metatranscriptomics and metaproteomics can measure actual expression of genetic potential but remains more technically difficult.

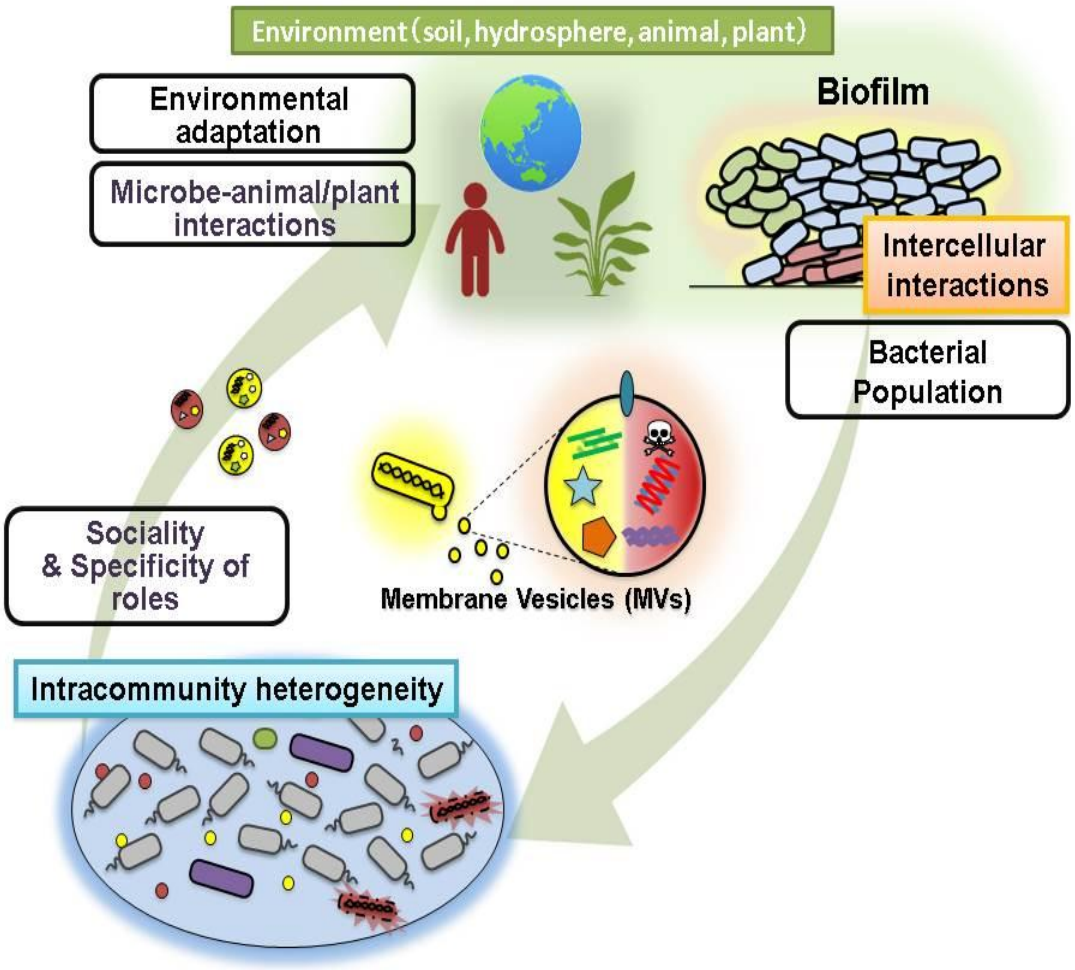
Roles

Microorganisms are the backbone of all ecosystems, but even more so in the zones where photosynthesis is unable to take place because of the absence of light. In such zones, chemosynthetic microbes provide energy and carbon to the other organisms. These chemotrophic organisms can also function in environments lacking oxygen by using other electron acceptors for their respiration.

Other microbes are decomposers, with the ability to recycle nutrients from other organisms' waste products. These microbes play a vital role in biogeochemical cycles The nitrogen cycle, the phosphorus cycle, the sulphur cycle and the carbon cycle all depend on microorganisms in one way or another.

Each cycle works together to regulate the microorganisms in certain processes. For example, the nitrogen gas which makes up 78% of the Earth's atmosphere is unavailable to most organisms, until it is converted to a biologically available form by the microbial process of nitrogen fixation. Differing from the nitrogen and carbon cycles, stable gaseous species are not created in the phosphorus cycle in the environment. Microorganisms play a role in solubilizing phosphate, improving soil health and plant growth.

Due to the high level of horizontal gene transfer among microbial communities,microbial ecology is also of importance to studies of evolution.



Microbial ecology and Role of microorganism in ecosystem

Evolution

Microbial ecology contributes to the evolution in many different parts of the world. For example, different microbial species evolved CRISPR dynamics and functions, allowing a better understanding of human health.

Symbiosis

Microbes, especially bacteria, often engage in symbiotic relationships (either positive or negative) with other microorganisms or larger organisms. Although physically small, symbiotic relationships amongst microbes are significant in eukaryotic processes and their evolution. The types of symbiotic relationship that microbes participate in include mutualism, commensalism, parasitism, and amensalism which affect the ecosystem in many ways.

Mutualism

Mutualism in microbial ecology is a relationship between microbial species and humans that allows for both sides to benefit. One such example would be syntrophy, also known as cross-feeding, of which 'Methanobacterium omelianskii is a classical example. This consortium is formed by an ethanol fermenting organism and a methanogen. The ethanol-fermenting organism provides the archaeal partner with the H2, which this methanogen needs in order to grow and produce methane.Syntrophy has been hypothesized to play a significant role in energy- and nutrient-limited environments, such as deep subsurface, where it can help the microbial community with diverse functional properties to survive, grow and produce maximum amount of energy. Anaerobic oxidation of methane (AOM) is carried out by mutualistic consortium of a sulfate-reducing bacterium and an anaerobic methane-oxidizing archaeon. The reaction used by the bacterial partner for the production of H_2 is endergonic (and so thermodynamically unfavored) however, when coupled to the reaction used by archaeal partner, the overall reaction becomes exergonic. Thus the two organisms are in a mutualistic relationship which allows them to grow and thrive in an environment, deadly for either species alone. Lichen is an example of a symbiotic organism.

Commensalism

Commensalism is very common in microbial world, literally meaning "eating from the same table". Metabolic products of one microbial population are used by another microbial population without either gain or harm for the first population. There are many "pairs "of microbial species that perform either oxidation or reduction reaction to the same chemical equation. For example, methanogens produce methane by reducing CO_2 to CH_4, while methanotrophs oxidise methane back to CO_2.

Amensalism

Amensalism (also commonly known as antagonism) is a type of symbiotic relationship where one species/organism is harmed while the other remains unaffected. One example of such a relationship that takes place in microbial ecology is between the microbial species Lactobacillus casei and Pseudomonas taetrolens. When co-existing in an environment, Pseudomonas taetrolens shows inhibited growth and decreased production of lactobionic acid (its main product) most likely due to the byproducts created by Lactobacillus casei during its production of lactic acid. However, Lactobacillus casei shows no difference in its behaviour, and such this relationship can be defined as amensalism.

Microbial resource management

Biotechnology may be used alongside microbial ecology to address a number of environmental and economic challenges. For example, molecular techniques such as community fingerprinting or metagenomics can be used to track changes in microbial communities over time or assess their biodiversity. Managing the carbon cycle to sequester carbon dioxide and prevent excess methanogenesis is important in mitigating global warming, and the prospects of bioenergy are being expanded by the development of microbial fuel cells. Microbial resource management advocates a more progressive attitude towards disease, whereby biological control agents are favoured over attempts at eradication. Fluxes in microbial communities has to be better characterized for this field's potential to be realised. In addition, there are also clinical implications, as marine microbial symbioses are a valuable source of existing and novel antimicrobial agents, and thus offer another line of inquiry in the evolutionary arms race of antibiotic resistance, a pressing concern for researchers.

In built environment and human interaction

Human microbiota

Microbes exist in all areas, including homes, offices, commercial centers, and hospitals. In 2016, the journal Microbiome published a collection of various works studying the microbial ecology of the built environment.

A 2006 study of pathogenic bacteria in hospitals found that their ability to survive varied by the type, with some surviving for only a few days while others survived for months.

The lifespan of microbes in the home varies similarly. Generally bacteria and viruses require a wet environment with humidity of over 10 percent. E. coli can survive for a few hours to a day. Bacteria which form spores can survive longer, with Staphylococcus aureus surviving potentially for weeks or, in the case of Bacillus anthracis, years.

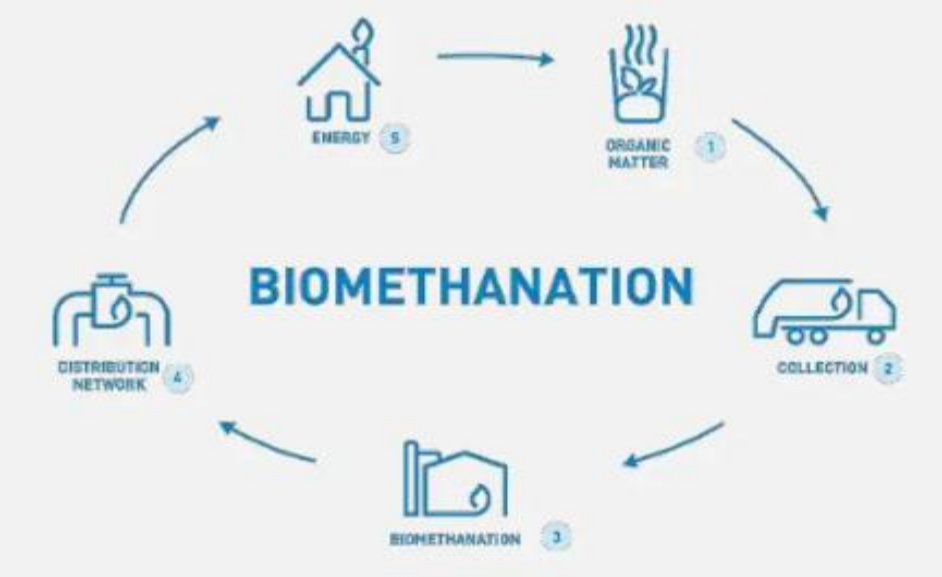
In the home, pets can be carriers of bacteria; for example, reptiles are commonly carriers of salmonella.

S. aureus is particularly common, and asymptomatically colonizes about 30% of the human population; attempts to decolonize carriers have met with limited success and generally involve mupirocin nasally and chlorhexidine washing, potentially along with vancomycin and cotrimoxazole to address intestinal and urinary tract infections.

Antimicrobials

Some metals, particularly copper, silver, and gold have antimicrobial properties. Using antimicrobial copper-alloy touch surfaces is a technique which has begun to be used in the 21st century to prevent transmission of bacteria. Silver nanoparticles have also begun to be incorporated into building surfaces and fabrics, although concerns have been raised about the potential side-effects of the tiny particles on human health. Due to the antimicrobial properties certain metals possess, products such as medical devices are made using those metals.

Biomethanation



A new high rate biomethanation technology for the integrated treatment of sewage and organic solid waste and concomitant generation of biogas and bio manure can treat groundwater and wastewater and convert it to potable water. It can be used for sewage and organic solid waste treatment with decentralized applications in various parts of India.

Solid and liquid wastes are major challenges faced by big cities as also for the rapidly urbanizing areas, and sustainable technologies are needed for tackling them.

An integrated treatment of sewage along with organic solid waste in India could help evolve a sustainable technology to overcome the solid and liquid waste disposal issues.

About

Biomethanation is a process by which organic material is microbiologically converted under anaerobic conditions to biogas.

Three main physiological groups of microorganisms are involved: fermenting bacteria, organic acid oxidizing bacteria, and methanogenic archaea.

Biogas

Biogas mainly consists of methane (about 60-75%), carbon dioxide (about 25-40%) besides small quantities of NH3 and H2S and has a Calorific Value of about 5000 kcal /m3.

Depending upon the waste composition, the biogas production ranges from 50-150m3/tonne of wastes.

The biogas can be utilised either for cooking / heating applications, or for generating motive power or electricity through dual-fuel, gas engines, low pressure gas turbines or steam turbines.

The sludge from anaerobic digestion, after stabilisation, can be used as a soil conditioner, or as manure depending upon its composition, which is determined mainly by the composition of the input waste.

Advantages of Anaerobic Digestion / Biomethanation

Generation of gaseous fuel;

Can be done on a small-scale;

No external power requirement unlike aerobic treatment;

Enclosed system enables all the gas produced to be collected for use. Green house gases Emission to the atmosphere is avoided;

Free from bad odour, rodent and fly menace, visible pollution and social resistance;

Modular construction of plant and closed treatment needs less land area; and

Production of biogas and high grade soil conditioner.

Disadvantages

In case of digesters operated under mesophilic temperatures, destruction of pathogenic organisms may be less than that in Aerobic Composting. However, several digester systems operated at high thermophilic temperatures are also available;

It is more capital intensive compared to composting and landfill; and

Not suitable for wastes containing less biodegradable matter.

Limitations/ Constraints

The growth of this sector has been affected on account of the following limitations/ constraints:

Waste-to-Energy is still a new concept in the country;

Most of the proven and commercial technologies in respect of urban wastes are required to be imported;

The costs of the projects especially based on biomethanation technology are high as critical equipment for a project is required to be imported.

In view of low level of compliance of MSW Rules 2000 by the Municipal Corporations/ Urban Local Bodies, segregated municipal solid waste is generally not available at the plant site, which may lead to non-availability of waste-to-energy plants.

Lack of financial resources with Municipal Corporations/Urban Local Bodies.

Lack of conducive Policy Guidelines from State Govts. in respect of allotment of land, supply of garbage and power purchase / evacuation facilities.

Final Thoughts

The underdeveloped state of waste management in India is a motivation for the study of eco-friendly processes like biomethanation and bioremediation.

The installation of biogas plants across various research institutes in India, like Sardar Patel Renewable Energy Research Institute (SPRERI) in Gujrat, Biogas Plant at Trombay, Appropriate Rural Technology Institute (ARTI) in Pune and Bhabha Atomic Research Centre (BARC) in Mumbai, practice biomethanation in a full-fledged process and yield high rate of biogas fuel from waste materials.

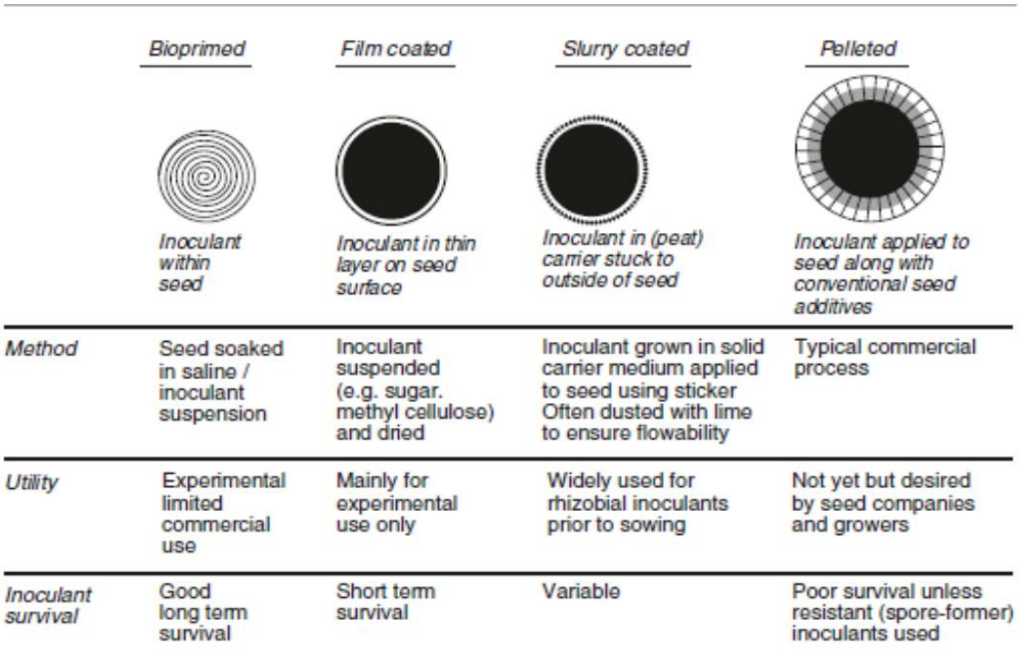
The biogas produced is clean, economical and used for commercial purposes.

But the government needs to invest more on this technology so that it percolates down to local bodies.

MICROBIAL EXPLOITATION FOR IMPROVEMENT AND CROP PROTECTION:

Human beings and animals are largely dependent on the plants for meeting their energy requirements. Also, continuous change in the environmental conditions due to climate change and global warming is having adverse effects on agricultural crops in commercial sectors. Conditions for plants become very drastic which are not suitable for their survival. So, at this stage, i.e., in the changing environment, it becomes essential to improve our agriculture system in such a way that we should not just aim for higher production but should also be looking for betterment in plant protection and adaptability, simultaneously. In the recent decade, we have seen huge increase in production in the agriculture sector which is largely attributed to the extensive use of synthetic chemicals, but this is not a long-term strategy for getting sustainable production. Due to high consumer pressure and new policies made by regulatory authorities, the withdrawal of these chemicals had been seen on a larger scale in order to reduce toxic residue in soil. In addition to this, the production, development, and registration cost for these synthetic chemicals has been inclining very rapidly, which is further limiting available control strategies for the growers. The pursuit for substitute solutions for agriculture has encouraged researchers to have a second look at the variety of microorganisms, recognized earlier to impart assistances to agricultural production, and is providing options for biocontrol agents and plant growth-promoting microbes In spite of the purpose for these microorganisms applied to crops, they must be produced at commercial or large scale and used in a way that maintains their activity and functionality in the target conditions also. So far, these formulations are available as liquids (sprays, drenches, root dips) mainly for experimentation or as dehydrated powder forms, which are supplied at the time of plantation. But most of these strategies do not work on mass production scale, due to a huge amount of microbial inoculum which is required. The beneficial microbes can be applied to the seeds for placing the microbial flora into the soil, where they will start colonizing and will provide protection by interacting against different insects and pests which are feeding on plant roots and reducing the yield. In addition, they will also synthesize essential nutrients which are required for proper growth and development of plants. This is not a new technique and is already established and demonstrated at small scale by different researchers in different conditions. However, despite the fact that they have been well demonstrated and available in various formulations in association with legume crops, they are still not used on commercial scale by the farmers due to lack of knowledge and interests.

Plant-microbiome interactions represent a very promising solution for providing protection and improving agricultural yield sustainably. In this chapter, we tried to merge the fundamental basics and applied aspects of beneficial plant-microbial interactions effectively. This is our humble approach and sincere effort for advancing the agriculture by providing details about available microbial solutions.



	Bioprimed	*Film coated*	*Slurry coated*	*Pelleted*
	Inoculant within seed	*Inoculant in thin layer on seed surface*	*Inoculant in (peat) carrier stuck to outside of seed*	*Inoculant applied to seed along with conventional seed additives*
Method	Seed soaked in saline / inoculant suspension	Inoculant suspended (e.g. sugar. methyl cellulose) and dried	Inoculant grown in solid carrier medium applied to seed using sticker Often dusted with lime to ensure flowability	Typical commercial process
Utility	Experimental limited commercial use	Mainly for experimental use only	Widely used for rhizobial inoculants prior to sowing	Not yet but desired by seed companies and growers
Inoculant survival	Good long term survival	Short term survival	Variable	Poor survival unless resistant (spore-former) inoculants used

Fig. 26.1 Methods and preparations available for microbial seed inoculation

Microorganisms for Sustainable Plant Growth

Plants are unsurprisingly accompanying multifaceted microbiomes, which are known to boost plant growth and stress tolerance, backing plant nutrition and antagonizing plant pathogens. The main properties of microorganisms for subsidiary plant growth and development.

Harmonizing Soil Ecology

Microorganisms are a fundamental part of almost every soil ecosystem. Soil is a hub of various kinds of biological and biochemical activities, and most of them are carried out by microorganisms. Metabolic activities of PGPR, mycorrhiza, cyanobacteria, and certain soil fauna have been reported to improve soil health and increase crop productivity. Most of the beneficial microorganisms need carbon as a sole source of energy, so that's why it has been observed that the soils which are poor in organic matter have less microbial activities. Moreover, the extensive use of synthetic fertilizers, insecticides, and pesticides in the last decades further lowered down the level of organic matter and

even worsens the situation by having adverse effects on microbialactivities. This led to decrease, or in many cases, a number of beneficial soil microorganisms such as PGPR, fungi, earthworms, actinomycetes, etc. have been extinct from those areas. Similar kind of situation prevails in almost all parts across the country.

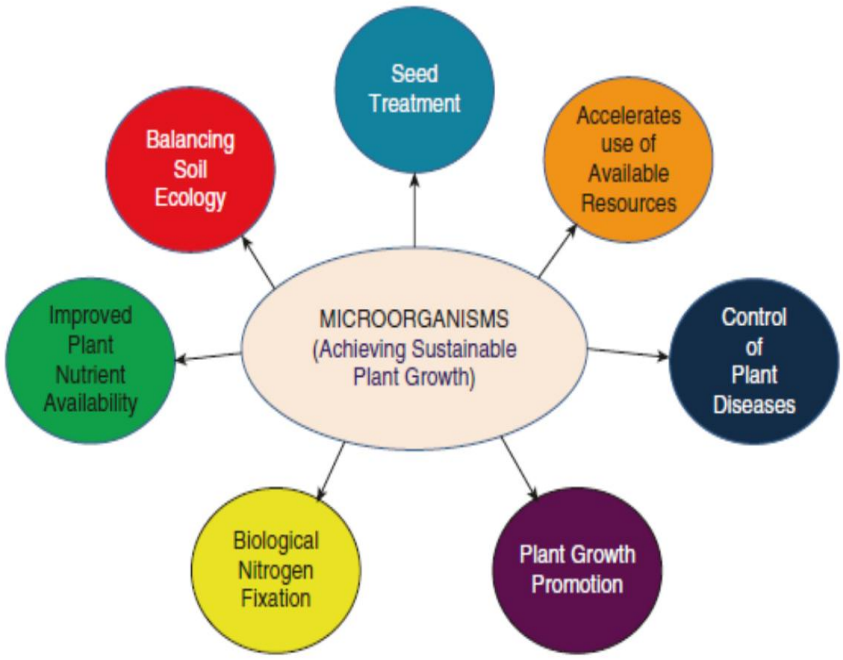


Fig. 26.2 Role of microorganism in plant growth

Biological Fixation of Nitrogen

Nitrogen is the most abundant gas on earth, but it is impossible to use nitrogen in its gaseous form by the plants. Also, nitrogen is a primary essential macro nutrient which is required by the plants and is a part of most of the biomolecules which has a role in physiological function and metabolism. There are many microorganisms which have the potential to convert gaseous nitrogen to its usable form that is nitrates through a process called as biological nitrogen fixation (BNF). BNF is the main process and source of nitrogen for legumes and other important crops. BNF provides the largest input of nitrogen to agricultural soils worldwide. Rhizobium inoculation as biofertilizer in the crops like groundnut, pigeon pea, soybean, etc. reported to provide 19–22 kg of nitrogen per hectare with 17–33% of total increase in crop yield. Likewise, the use of Azotobacter, which is a nonsymbiotic bacterium, and Azospirillum in wheat, sorghum, tomato, cotton, and sugarcane contributed nitrogen supply to crops to an extent of 20–30 kg per hectare providing 10–30% increased crop yield.

Wherever water, sunlight, and carbon dioxide are available, phototrophic microorganisms like blue-green algae or cyanobacteria can grow. Therefore, rice ecosystem provides an ideal environment for the growth and development of these self-supporting organisms such as Anabaena, Nostoc, Aulosira, Calothrix, Tolypothrix, etc. They colonize the rice field soils, compete well with the native strains, thus grow profusely near the rhizosphere, and release fixed nitrogen through exudation or through microbial decomposition after the algae dies. So, in rice fields, the degradation of algal biomass most frequently results in maintenance of soil fertility. The residual effects influence the succeeding crops also. Apart from fixing nitrogen and adding organic matter to soil, BGA are also known to produce and excrete plant promoting substances like indoleacetic acid. Also, the continuous use of the BGA biofertilizers for 2–3 years adequately builds up the population of these organisms in the soil.

The relative contribution of **BGA** as a percentage of total nitrogen fixed in paddy fields varies widely and is estimated to be 15–35 kg nitrogen per hectare in India. In areas where chemical nitrogen is not used for various reasons, algal inoculation enhances minimum of 4% to a maximum of 32.8% crop yield with an overall average of 16.1%. Even at the levels of chemical nitrogen fertilizers being used in different states, the application of BGA biofertilizers resulted in an increased crop yield of 8.85%. Plant growth-promoting rhizobacteria (PGPR) are low-cost input from nature; besides nitrogen fixers, many bacteria colonize plant roots. Some of them promote plant growth significantly. They help in mobilization of the soil nutrients and production of phytohormones or growth-regulating substances. These phytohormone producing microbes have been classified as PGPR. Of the many such bacteria identified, the role of fluorescent Pseudomonas and Bacillus species has attracted much attention. The substances produced by them have natural biocontrol and plant growth-promoting capabilities. Increased amount of nutrient uptake by plants inoculated by Pseudomonas putida has been attributed to the production of growth regulators by the bacterium at root surface which stimulates root development. Pseudomonad (group of Pseudomonas sp.) inoculants produce indoleacetic acidlike substances (plant hormone) in the rhizosphere of wheat grown in field conditions. Many **PGPR**, for example, Pseudomonas fluorescens, Pseudomonas aeruginosa, and Bacillus subtilis, also produce substances such as siderophores and saponins, which are responsible for the removal of heavy metal toxicity. These organisms are also responsible for enhancement of rhizospheric competitive ability by antagonistic effects on other harmful bacteria; control of plant diseases that affect root density; and production of chemicals that interfere with the organisms infecting plant roots, enhancing the availability of nutrients that improve the efficacy of plants.

PGPR are therefore being widely evaluated for their role in sustainable resource management as biocontrol agent and Biofertilizer.

Table 26.1 Growth-promoting substances released by some important plant growth-promoting bacteria

Beneficial bacteria	Growth-promoting properties/compounds
Pseudomonas putida	Siderophores, IAA, ammonia, phosphate solubilization, HCN, exo-polysaccharides
Pseudomonas aeruginosa	Siderophores, IAA, ammonia, phosphate solubilization, HCN, exo-polysaccharides
Klebsiella sp.	Siderophores, IAA, ammonia, phosphate solubilization, HCN, exo-polysaccharides
Enterobacter asburiae	Siderophores, IAA, ammonia, phosphate solubilization, HCN, exo-polysaccharides
Mesorhizobium sp.	HCN, ammonia, IAA, exo-polysaccharides, siderophores
Acinetobacter sp.	Phosphate solubilization, IAA, siderophores
Rhizobium sp.(pea)	HCN, ammonia, IAA, exo-polysaccharides, siderophores
Rhizobium sp.(lentil)	HCN, ammonia, IAA, exo-polysaccharides, siderophores
Pseudomonas sp. A3R3	IAA, siderophores
Psychrobacter sp. SRS8	Heavy metal mobilization
Bradyrhizobium sp.	HCN, ammonia, IAA, exo-polysaccharides, siderophores
Pseudomonas aeruginosa 4EA	Siderophores
Bradyrhizobium sp.750	Heavy metal mobilization
Bacillus species PSB10	Ammonia, IAA, siderophores, HCN
Paenibacillus polymyxa	Siderophores, IAA
Rhizobium phaseoli	IAA
Stenotrophomonas maltophilia	Nitrogenase activity, phosphate solubilization, IAA, ACC deaminase
Rahnella aquatilis	ACC deaminase, phosphate solubilization, IAA
Proteus vulgaris	Siderophores
Pseudomonas sp.	Siderophore, phosphate solubilization, IAA
Azospirillum amazonense	Biocontrol potentials, nitrogenase activity, HCN, IAA
Mesorhizobium sp.	IAA, siderophores, HCN, ammonia
Pseudomonas sp.	ACC deaminase, IAA, siderophore
Serratia marcescens	IAA, siderophore, HCN
Pseudomonas fluorescens	ACC deaminase, phosphate solubilization
Enterobacter sp.	Phosphate solubilization, siderophore, N2 fixation, phosphate solubilization, ACC deaminase, IAA

IAA indole-3-acetic acid, *HCN* hydrogen cyanide, *ACC* 1-aminocyclopropane-1-carboxylate

Mycorrhizae play a dominant role in making unavailable soil nutrients available to plant roots and increasing the potential gain of available resources. These organisms ensure easy availability of organic carbon and complex organic nitrogen and phosphorus sources and increase phosphorus solubilization and availability in clay soils. These fungi work upon large volumes of soil. Their hyphae extend outwardly from the roots ranging from a few centimeters to several meters in the soil.

This results in increasing the effective absorbing surface of the host root by as much as ten times, resulting in enhanced absorption of immobile nutrients such as phosphorus, zinc, copper, etc. in the soil by 60 times.

Mycorrhizal fungi also transport many other nutrients including calcium, magnesium, sodium, sulfur, iron, chlorine, etc., all essential for plant growth and development. It has been reported that plants with mycorrhizal association are more tolerant to heavy metal toxicity. These plants survive well in drought and arid conditions as improved water movement is facilitated by mycorrhiza. Theoretically, the most efficient level of nutrients is the concentration of mineral elements in the plant tissue just above the "critical level" necessary for optimum growth. Further addition of chemical fertilizers may be taken up by plants, as "luxury concentration." This adds very little to plant growth. Now, these microorganisms help in constituting the "optimum level" of minerals in the plant tissue even at low level of fertilizer inputs. They fix nitrogen, solubilize phosphorus, and facilitate uptake of minerals by roots. Thus, these microorganisms in the form of biofertilizers are essential for maintaining good soil fertility, better soil conditions, and sustainable agricultural productivity.

Seed Treatments

There is a growing curiosity in the use of soil microorganisms which are beneficial for plant development as potential substitutes to synthetic fertilizers and pesticides in agricultural production. Seed inoculation techniques developed for research purposes are often not possible to be implemented at a commercial scale because of significant obstacles or challenges like technical aspects for maintaining viable microbial inocula throughout complete seed treatment process and seed storage. Further research advances in these technologies are required for imparting benefits of a wide range of environmentally sensitive potential seed inoculants in the field of agriculture. Presently, there are no solutions available for commercialization of seed inoculation treatments at commercial scale. So, there is an urgent need for association of scientific fields like soil science, microbiology, biotechnology, agriculture, and adjuvant chemistry to develop a sustainable protocol for making these technologies commercially viable and available to farmers.

Control of Plant Diseases and Plant Growth Promotion

Seeds are exposed to fungicides and bactericides in order to prevent crop failure because of seedor soil-borne pathogens. Normally these treatments are chemical techniques which are cytotoxic; it means they can also have adverse effects on the viability of seeds and their germination potential.

Microbial inocula, which are antagonistic to soil-borne pathogens, is an ideal delivery system as it directly introduces inoculum to the rhizosphere of the plant where plant pathogens like Pythium and Rhizoctonia are active, causing diseases like seed rots in the spermosphere and damping-off disease in seedling. Various bacterial and fungal antagonists have been identified and developed experimentally and commercially for this purpose, but their use as seed treatments is still very limited.

Biological control of plant diseases and agricultural antibiotics

Introduction

Plant diseases need to be controlled to maintain the quality and abundance of food, feed, and fiber produced by growers around the world. Different approaches may be used to prevent, mitigate or control plant diseases. Beyond good agronomic and horticultural practices, growers often rely heavily on chemical fertilizers and pesticides. Such inputs to agriculture have contributed significantly to the spectacular improvements in crop productivity and quality over the past 100 years. However, the environmental pollution caused by excessive use and misuse of agrochemicals, as well as fear-mongering by some opponents of pesticides, has led to considerable changes in people's attitudes towards the use of pesticides in agriculture. Today, there are strict regulations on chemical pesticide use, and there is political pressure to remove the most hazardous chemicals from the market. Additionally, the spread of plant diseases in natural ecosystems may preclude successful application of chemicals, because of the scale to which such applications might have to be applied. Consequently, some pest management researchers have focused their efforts on developing alternative inputs to synthetic chemicals for controlling pests and diseases. Among these alternatives are those referred to as biological controls.

A variety of biological controls are available for use, but further development and effective adoption will require a greater understanding of the complex interactions among plants, people, and the environment. To that end, this article is presented as an advanced survey of the nature and practice of biological control as it is applied to the suppression of plant diseases. This survey will i) describe the various definitions and key mechanisms of biocontrol, ii) explore the relationships between microbial diversity and biological control, iii) describe the current status of research and application of biological controls, and iv) briefly outline future directions that might lead to the development of more diverse and effective biological controls for plant diseases.

Definitions

The terms “biological control” and its abbreviated synonym “biocontrol” have been used in different fields of biology, most notably entomology and plant pathology. In entomology, it has been used to describe the use of live predatory insects, entomopathogenic nematodes, or The Plant Health Instructor, microbial pathogens to suppress populations of different pest insects. In plant pathology, the term applies to the use of microbial antagonists to suppress diseases as well as the use of hostspecific pathogens to control weed populations. In both fields, the organism that suppresses the pest or pathogen is referred to as the biological control agent (BCA). More broadly, the term biological control also has been applied to the use of the natural products extracted or fermented from various sources. These formulations may be very simple mixtures of natural ingredients with specific activities or complex mixtures with multiple effects on the host as well as the target pest or pathogen. And, while such inputs may mimic the activities of living organisms, nonliving inputs should more properly be referred to as biopesticides or biofertilizers, depending on the primary benefit provided to the host plant. The various definitions offered in the scientific literature have sometimes caused confusion and controversy. For example, members of the U.S. National Research Council took into account modern biotechnological developments and referred to biological control as “the use of natural or modified organisms, genes, or gene products, to reduce the effects of undesirable organisms and to favor desirable organisms such as crops, beneficial insects, and microorganisms”, but this definition spurred much subsequent debate and it was frequently considered too broad by many scientists who worked in the field. Because the term biological control can refer to a spectrum of ideas, it is important to stipulate the breadth of the term when it is applied to the review of any particular work.

Published definitions of biocontrol differ depending on the target of suppression; number, type and source of biological agents; and the degree and timing of human intervention. Most broadly, biological control is the suppression of damaging activities of one organism by one or more other organisms, often referred to as natural enemies. With regards to plant diseases, suppression can be accomplished in many ways. If growers’ activities are considered relevant, cultural practices such as the use of rotations and planting of disease resistant cultivars (whether naturally selected or genetically engineered) would be included in the definition. Because the plant host responds to numerous biological factors, both pathogenic and non-pathogenic, induced host resistance might be considered a form of biological control. More narrowly, biological control refers to the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens.

This may involve the use of microbial inoculants to suppress a single type or class of plant diseases. Or, this may involve managing soils to promote the combined activities of native soil- and plant-associated organisms that contribute to general suppression. Most narrowly, biological control refers to the suppression of a single pathogen (or pest), by a single antagonist, in a single cropping system. Most specialists in the field would concur with one of the narrower definitions presented above. In this review, biological control will be narrowly defined as highlighted above in bold.

Types of interactions contributing to biological control

Throughout their lifecycle, plants and pathogens interact with a wide variety of organisms. These interactions can significantly affect plant health in various ways. In order to understand the mechanisms of biological control, it is helpful to appreciate the different ways that organisms interact. Note, too, that in order to interact, organisms must have some form of direct or indirect contact. Odum (1953) proposed that the interactions of two populations be defined by the outcomes for each. The types of interactions were referred to as mutualism, protocooperation, commensalism, neutralism, competition, amensalism, parasitism, and predation. While the terminology was developed for macroecology, examples of all of these types of interactions can be found in the natural world at both the macroscopic and microscopic level. And, because the development of plant diseases involves both plants and microbes, the interactions that lead to biological control take place at multiple levels of scale.

From the plant's perspective, biological control can be considered a net positive result arising from a variety of specific and non-specific interactions. Using the spectrum of Odum's concepts, we can begin to classify and functionally delineate the diverse components of ecosystems that contribute to biocontrol. Mutualism is an association between two or more species where both species derive benefit. Sometimes, it is an obligatory lifelong interaction involving close physical and biochemical contact, such as those between plants and mycorrhizal fungi. However, they are generally facultative and opportunistic. For example, bacteria in the genus Rhizobium can reproduce either in the soil or, to a much greater degree, through their mutualistic association with legume plants. These types of mutualism can contribute to biological control, by fortifying the plant with improved nutrition and/or by stimulating host defenses. Protocooperation is a form of mutualism, but the organisms involved do not depend exclusively on each other for survival. Many of the microbes isolated and classified as BCAs can be considered facultative mutualists involved in protocooperation, because survival rarely depends on any specific host and disease suppression will vary depending on the prevailing environmental conditions.

Further down the spectrum, commensalism is a symbiotic interaction between two living organisms, where one organism benefits and the other is neither harmed nor benefited. Most plant-associated microbes are assumed to be commensals with regards to the host plant, because their presence, individually or in total, rarely results in overtly positive or negative consequences to the plant. And, while their presence may present a variety of challenges to an infecting pathogen, an absence of measurable decrease in pathogen infection or disease severity is indicative of commensal interactions. Neutralism describes the biological interactions when the population density of one species has absolutely no effect whatsoever on the other. Related to biological control, an inability to associate the population dynamics of pathogen with that of another organism would indicate neutralism. In contrast, antagonism between organisms results in a negative outcome for one or both. Competition within and between species results in decreased growth, activity and/or fecundity of the interacting organisms. Biocontrol can occur when non-pathogens compete with pathogens for nutrients in and around the host plant. Direct interactions that benefit one population at the expense of another also affect our understanding of biological control. Parasitism is a symbiosis in which two phylogenetically unrelated organisms coexist over a prolonged period of time. In this type of association, one organism, usually the physically smaller of the two (called the parasite) benefits and the other (called the host) is harmed to some measurable extent. The activities of various hyperparasites, i.e., those agents that parasitize plant pathogens, can result in biocontrol. And, interestingly, host infection and parasitism by relatively avirulent pathogens may lead to biocontrol of more virulent pathogens through the stimulation of host defense systems. Lastly, predation refers to the hunting and killing of one organism by another for consumption and sustenance. While the term predator typically refer to animals that feed at higher trophic levels in the macroscopic world, it has also been applied to the actions of microbes, e.g. protists, and mesofauna, e.g. fungal feeding nematodes and microarthropods, that consume pathogen biomass for sustenance. Biological control can result in varying degrees from all of these types of interactions, depending on the environmental context within which they occur. Significant biological control, as defined above, most generally arises from manipulating mutualisms between microbes and their plant hosts or from manipulating antagonisms between microbes and pathogens.

Mechanisms of biological control

Because biological control can result from many different types of interactions between organisms, researchers have focused on characterizing the mechanisms operating in different experimental situations. In all cases, pathogens are antagonized by the presence and activities of other organisms that they encounter.

Here, we assert that the different mechanisms of antagonism occur across a spectrum of directionality related to the amount of interspecies contact and specificity of the interactions. Direct antagonism results from physical contact and/or a high-degree of selectivity for the pathogen by the mechanism(s) expressed by the BCA(s). In such a scheme, hyperparasitism by obligate parasites of a plant pathogen would be considered the most direct type of antagonism because the activities of no other organism would be required to exert a suppressive effect. In contrast, indirect antagonisms result from activities that do not involve sensing or targeting a pathogen by the BCA(s).

Types of interspecies antagonisms leading to biological control of plant pathogens.

Type	Mechanism	Examples
Direct antagonism	Hyperparasitism/predation	Lytic/some nonlytic mycoviruses *Ampelomyces quisqualis* *Lysobacter enzymogenes* *Pasteuria penetrans* *Trichoderma virens*
Mixed-path antagonism	Antibiotics	2,4-diacetylphloroglucinol Phenazines Cyclic lipopeptides
	Lytic enzymes	Chitinases Glucanases Proteases
	Unregulated waste products	Ammonia Carbon dioxide Hydrogen cyanide
	Physical/chemical interference	Blockage of soil pores Germination signals consumption Molecular cross-talk confused
Indirect antagonism	Competition	Exudates/leachates consumption Siderophore scavenging Physical niche occupation
	Induction of host resistance	Contact with fungal cell walls Detection of pathogen-associated, molecular patterns Phytohormone-mediated induction

Stimulation of plant host defense pathways by non-pathogenic BCAs is the most indirect form of antagonism. However, in the context of the natural environment, most described mechanisms of pathogen suppression will be modulated by the relative occurrence of other organisms in addition to the pathogen. While many investigations have attempted to establish the importance of specific mechanisms of biocontrol to particular pathosystems, all of the mechanisms described below are likely to be operating to some extent in all natural and managed ecosystems.

And, the most effective BCAs studied to date appear to antagonize pathogens using multiple mechanisms. For instance, pseudomonads known to produce the antibiotic 2,4-diacetylphloroglucinol (DAPG) may also induce host defenses (Iavicoli et al. 2003). Additionally, DAPG-producers can aggressively colonize roots, a trait that might further contribute to their ability to suppress pathogen activity in the rhizosphere of wheat through competition for organic nutrients.

Hyperparasites and predation

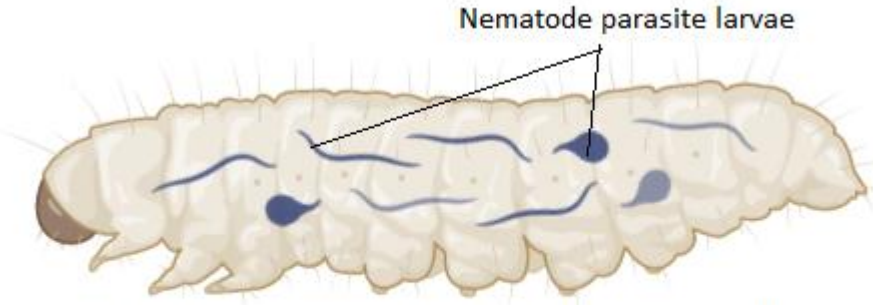
In hyperparasitism, the pathogen is directly attacked by a specific BCA that kills it or its propagules. In general, there are four major classes of hyperparasites: obligate bacterial pathogens, hypoviruses, facultative parasites, and predators. Pasteuria penetrans is an obligate bacterial pathogen of root-knot nematodes that has been used as a BCA. Hypoviruses are hyperparasites. A classical example is the virus that infects Cryphonectria parasitica, a fungus causing chestnut blight, which causes hypovirulence, a reduction in disease-producing capacity of the pathogen. The phenomenon has controlled the chestnut blight in many places . However, the interaction of virus, fungus, tree, and environment determines the success or failure of hypovirulence. There are several fungal parasites of plant pathogens, including those that attack sclerotia (e.g. Coniothyrium minitans) while others attack living hyphae (e.g. Pythium oligandrum). And, a single fungal pathogen can be attacked by multiple hyperparasites. For example, Acremonium alternatum, Acrodontium crateriforme, Ampelomyces quisqualis, Cladosporium oxysporum, and Gliocladium virens are just a few of the fungi that have the capacity to parasitize powdery mildew pathogens. Other hyperparasites attack plant-pathogenic nematodes during different stages of their life cycles (e.g. Paecilomyces lilacinus and Dactylella oviparasitica). In contrast to hyperparasitism, microbial predation is more general and pathogen non-specific and generally provides less predictable levels of disease control. Some BCAs exhibit predatory behavior under nutrient-limited conditions. However, such activity generally is not expressed under typical growing conditions. For example, some species of Trichoderma produce a range of enzymes that are directed against cell walls of fungi. However, when fresh bark is used in composts, *Trichoderma* spp. does not directly attack the plant pathogen, *Rhizoctonia solani*. But in decomposing bark, the concentration of readily available cellulose decreases and this activates the chitinase genes of Trichoderma spp., which in turn produce chitinase to parasitize R. solani.

Antibiotic-mediated suppression Antibiotics are microbial toxins that can, at low concentrations, poison or kill other microorganisms. Most microbes produce and secrete one or more compounds with antibiotic activity.

In some instances, antibiotics produced by microorganisms have been shown to be particularly effective at suppressing plant pathogens and the diseases they cause.

Table 2. Some of antibiotics produced by BCAs

Antibiotic	Source	Target pathogen	Disease	Reference
2, 4-diacetyl-phloroglucinol	*Pseudomonas fluorescens* F113	*Pythium spp.*	Damping off	Shanahan et al. (1992),
Agrocin 84	*Agrobacterium radiobacter*	*Agrobacterium tumefaciens*	Crown gall	Kerr (1980)
Bacillomycin D	*Bacillus subtilis* AU195	*Aspergillus flavus*	Aflatoxin contamination	Moyne et al. (2001)
Bacillomycin, fengycin	*Bacillus amyloliquefaciens* FZB42	*Fusarium oxysporum*	Wilt	Koumoutsi et al. (2004)
Xanthobaccin A	*Lysobacter* sp. strain SB-K88	*Aphanomyces cochlioides*	Damping off	Islam et al. (2005)
Gliotoxin	*Trichoderma virens*	*Rhizoctonia solani*	Root rots	Wilhite et al. (2001)
Herbicolin	*Pantoea agglomerans* C9-1	*Erwinia amylovora*	Fire blight	Sandra et al. (2001)
Iturin A	*B. subtilis* QST713	*Botrytis cinerea* and *R. solani*	Damping off	Paulitz and Belanger (2001), Kloepper et al. (2004)
Mycosubtilin	*B. subtilis* BBG100	*Pythium aphanidermatum*	Damping off	Leclere et al. (2005)
Phenazines	*P. fluorescens* 2-79 and 30-84	*Gaeumannomyces graminis* var. *tritici*	Take-all	Thomashow et al. (1990)
Pyoluteorin, pyrrolnitrin	*P. fluorescens* Pf-5	*Pythium ultimum* and *R. solani*	Damping off	Howell and Stipanovic (1980)
Pyrrolnitrin, pseudane	*Burkholderia cepacia*	*R. solani* and *Pyricularia oryzae*	Damping off and rice blast	Homma et al. (1989)
Zwittermicin A	*Bacillus cereus* UW85	*Phytophthora medicaginis* and *P. aphanidermatum*	Damping off	Smith et al. (1993)



An Entomogenous Nematode hyperparasite living inside a worm parasite of plants

Hyperparasite is a parasite which A. kills its hostB. Completes the life cycle in one hostC. Uses host machinery for reproductionD

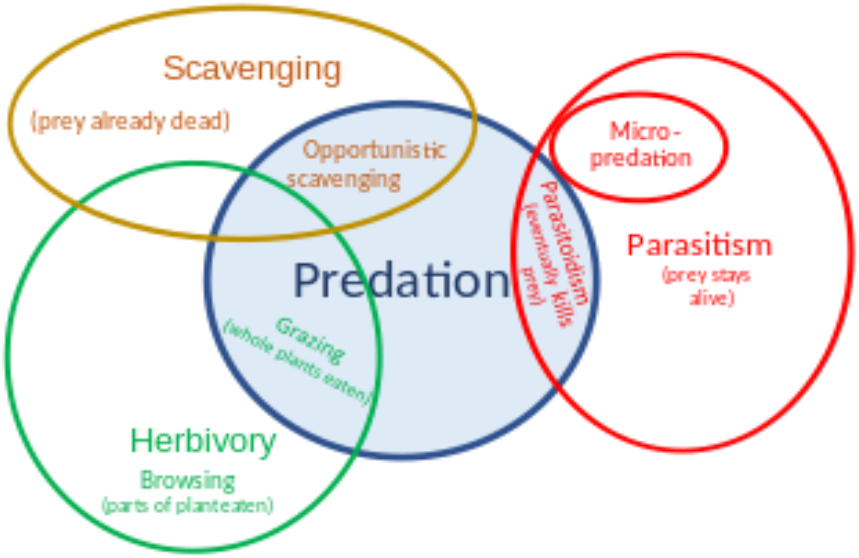
Predation is a biological interaction where one organism, the **predator**, kills and eats another organism, its **prey**. It is one of a family of common feeding behaviours that includes parasitism and micropredation (which usually do not kill the host) and parasitoidism (which always does, eventually). It is distinct from scavenging on dead prey, though many predators also scavenge; it overlaps with herbivory, as seed predators and destructive frugivores are predators.

Predators may actively search for or pursue prey or wait for it, often concealed. When prey is detected, the predator assesses whether to attack it. This may involve ambush or pursuit predation, sometimes after stalking the prey. If the attack is successful, the predator kills the prey, removes any inedible parts like the shell or spines, and eats it.

Predators are adapted and often highly specialized for hunting, with acute senses such as vision, hearing, or smell. Many predatory animals, both vertebrate and invertebrate, have sharp claws or jaws to grip, kill, and cut up their prey. Other adaptations include stealth and aggressive mimicry that improve hunting efficiency.

Predation has a powerful selective effect on prey, and the prey develop antipredator adaptations such as warning coloration, alarm calls and other signals, camouflage, mimicry of well-defended species, and defensive spines and chemicals. Sometimes predator and prey find themselves in an evolutionary arms race, a cycle of adaptations and counter-adaptations. Predation has been a major driver of evolution since at least the Cambrian period.

At the most basic level, predators kill and eat other organisms. However, the concept of predation is broad, defined differently in different contexts, and includes a wide variety of feeding methods; and some relationships that result in the prey's death are not generally called predation. A parasitoid, such as an ichneumon wasp, lays its eggs in or on its host; the eggs hatch into larvae, which eat the host, and it inevitably dies. Zoologists generally call this a form of parasitism, though conventionally parasites are thought not to kill their hosts. A predator can be defined to differ from a parasitoid in that it has many prey, captured over its lifetime, where a parasitoid's larva has just one, or at least has its food supply provisioned for it on just one occasion.



Relation of predation to other feeding strategies

There are other difficult and borderline cases. Micropredators are small animals that, like predators, feed entirely on other organisms; they include fleas and mosquitoes that consume blood from living animals, and aphids that consume sap from living plants. However, since they typically do not kill their hosts, they are now often thought of as parasites. Animals that graze on phytoplankton or mats of microbes are predators, as they consume and kill their food organisms; but herbivores that browse leaves are not, as their food plants usually survive the assault.When animals eat seeds (*seed predation* or *granivory*) or eggs (*egg predation*), they are consuming entire living organisms, which by definition makes them predators.

Scavengers, organisms that only eat organisms found already dead, are not predators, but many predators such as the jackal and the hyena scavenge when the opportunity arises. Among invertebrates, social wasps (yellowjackets) are both hunters and scavengers of other insects.

Some examples of antibiotics reported to be involved in plant pathogen suppression are listed

Quantities near the pathogen to result in a biocontrol effect. In situ production of antibiotics by several different biocontrol agents has been measured; however, the effective quantities are difficult to estimate because of the small quantities produced relative to the other, less toxic, organic compounds present in the phytosphere. And while methods have been developed to ascertain when and where biocontrol agents may produce antibiotics, detecting expression in the infection court is difficult because of the heterogenous distribution of plant-associated microbes and the potential sites of infection. In a few cases, the relative importance of antibiotic production by biocontrol bacteria has been demonstrated, where one or more genes responsible for biosynthesis of the

antibiotics have been manipulated. For example, mutant strains incapable of producing phenazines or phloroglucinols have been shown to be equally capable of colonizing the rhizosphere but much less capable of suppressing soilborne root diseases than the corresponding wild-type and complemented mutant strains. Several biocontrol strains are known to produce multiple antibiotics which can suppress one or more pathogens. For example, Bacillus cereus strain UW85 is known to produce both zwittermycin and kanosamine. The ability to produce multiple antibiotics probably helps to suppress diverse microbial competitors, some of which are likely to be plant pathogens. The ability to produce multiple classes of antibiotics, that differentially inhibit different pathogens, is likely to enhance biological control. More recently, Pseudomonas putida WCS358r strains genetically engineered to produce phenazine and DAPG displayed improved capacities to suppress plant diseases in field-grown wheat.

Lytic enzymes and other byproducts of microbial life

Diverse microorganisms secrete and excrete other metabolites that can interfere with pathogen growth and/or activities. Many microorganisms produce and release lytic enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemicellulose, and DNA. Expression and secretion of these enzymes by different microbes can sometimes result in the suppression of plant pathogen activities directly. For example, control of Sclerotium rolfsii by Serratia marcescens appeared to be mediated by chitinase expression. And, a b-1,3-glucanase contributes significantly to biocontrol activities of Lysobacter enzymogenes strain C3 . While they may stress and/or lyse cell walls of living organisms, these enzymes generally act to decompose plant residues and nonliving organic matter. Currently, it is unclear how much of the lytic enzyme activity that can be detected in the natural environment represents specific responses to microbe-microbe interactions. It seems more likely that such activities are largely indicative of the need to degrade complex polymers in order to obtain carbon nutrition. Nonetheless, microbes that show a preference for colonizing and lysing plant pathogens might be classified as biocontrol agents. Lysobacter and Myxobacteria are known to produce copious amounts of lytic enzymes, and some isolates have been shown to be effective at suppressing fungal plant pathogens. So, the lines between competition, hyperparasitism, and antibiosis are generally blurred. Furthermore, some products of lytic enzyme activity may contribute to indirect disease suppression. For example, oligosaccharides derived from fungal cell walls are known to be potent inducers of plant host defenses. Interestingly, Lysobacter enzymogenes strain C3 has been shown to induce plant host resistance to, though the precise activities leading to this induction are not entirely clear.

The quantitative contribution of any and all of the above compounds to disease suppression is likely to be dependent on the composition and carbon to nitrogen ratio of the soil organic matter that serves as a food source for microbial populations in the soil and rhizosphere. However, such activities can be manipulated so as to result in greater disease suppression. For example, in postharvest disease control, addition of chitosan can stimulate microbial degradation of pathogens similar to that of an applied hyperparasite. Chitosan is a non-toxic and biodegradable polymer of beta-1,4-glucosamine produced from chitin by alkaline deacylation. Amendment of plant growth substratum with chitosan suppressed the root rot caused by Fusarium oxysporum f. sp. radicis-lycopersici in tomato. Although the exact mechanism of action of chitosan is not fully understood, it has been observed that treatment with chitosan increased resistance to pathogens.

Other microbial byproducts also may contribute to pathogen suppression. Hydrogen cyanide (HCN) effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. The production of HCN by certain fluorescent pseudomonads is believed to be involved in the suppression of root pathogens. P. fluorescens CHA0 produces antibiotics, siderophores and HCN, but suppression of black rot of tobacco caused by Thielaviopsis basicola appeared to be due primarily to HCN production . Howell et al. (1988) reported that volatile compounds such as ammonia produced by Enterobacter cloacae were involved in the suppression of Pythium ultimum-induced damping-off of cotton. While it is clear that biocontrol microbes can release many different compounds into their surrounding environment, the types and amounts produced in natural systems in the presence and absence of plant disease have not been well documented and this remains a frontier for discovery.

Competition

From a microbial perspective, soils and living plant surfaces are frequently nutrient limited environments. To successfully colonize the phytosphere, a microbe must effectively compete for the available nutrients. On plant surfaces, host-supplied nutrients include exudates, leachates, or senesced tissue. Additionally, nutrients can be obtained from waste products of other organisms such as insects (e.g. aphid honeydew on leaf surface) and the soil. While difficult to prove directly, much indirect evidence suggests that competition between pathogens and non-pathogens for nutrient resources is important for limiting disease incidence and severity. In general, soilborne pathogens, such as species of Fusarium and Pythium, that infect through mycelial contact are more susceptible to competition from other soil- and plant-associated microbes than those pathogens that germinate directly on plant surfaces and infect through appressoria and infection pegs.

Genetic work of Anderson et al. (1988) revealed that production of a particular plant glycoprotein called agglutinin was correlated with potential of P. putida to colonize the root system. P. putida mutants deficient in this ability exhibited reduced capacity to colonize the rhizosphere and a corresponding reduction in Fusarium wilt suppression in cucumber. The most abundant nonpathogenic plant-associated microbes are generally thought to protect the plant by rapid colonization and thereby exhausting the limited available substrates so that none are available for pathogens to grow. For example, effective catabolism of nutrients in the spermosphere has been identified as a mechanism contributing to the suppression of Pythium ultimum by Enterobacter cloacae. At the same time, these microbes produce metabolites that suppress pathogens. These microbes colonize the sites where water and carboncontaining nutrients are most readily available, such as exit points of secondary roots, damaged epidermal cells, and nectaries and utilize the root mucilage.

Biocontrol based on competition for rare but essential micronutrients, such as iron, has also been examined. Iron is extremely limited in the rhizosphere, depending on soil pH. In highly oxidized and aerated soil, iron is present in ferric form, which is insoluble in water (pH 7.4) and the concentration may be as low as 10-18 M. This concentration is too low to support the growth of microorganisms, which generally need concentrations approaching 10-6 M. To survive in such an environment, organisms were found to secrete iron-binding ligands called siderophores having high affinity to sequester iron from the micro-environment. Almost all microorganisms produce siderophores, of either the catechol type or hydroxamate type. Kloepper et al. (1980) were the first to demonstrate the importance of siderophore production as a mechanism of biological control of Erwinia carotovora by several plant-growthpromoting Pseudomonas fluorescens strains A1, BK1, TL3B1 and B10. And, a direct correlation was established in vitro between siderophore synthesis in fluorescent pseudomonads and their capacity to inhibit germination of chlamydospores of F. oxysporum. As with the antibiotics, mutants incapable of producing some siderophores, such as pyoverdine, were reduced in their capacity to suppress different plant pathogens . The increased efficiency in iron uptake of the commensal microorganisms is thought to be a contributing factor to their ability to aggressively colonize plant roots and an aid to the displacement of the deleterious organisms from potential sites of infection.

Induction of host resistance

Plants actively respond to a variety of environmental stimuli, including gravity, light, temperature, physical stress, water and nutrient availability. Plants also respond to a variety of chemical stimuli produced by soil- and plant-associated microbes.

Such stimuli can either induce or condition plant host defenses through biochemical changes that enhance resistance against subsequent infection by a variety of pathogens. Induction of host defenses can be local and/or systemic in nature, depending on the type, source, and amount of stimuli. Recently, phytopathologists have begun to characterize the determinants and pathways of induced resistance stimulated by biological control agents and other non-pathogenic microbes. The first of these pathways, termed systemic acquired resistance (SAR), is mediated by salicylic acid (SA), a compound which is frequently produced following pathogen infection and typically leads to the expression of pathogenesis-related (PR) proteins. These PR proteins include a variety of enzymes some of which may act directly to lyse invading cells, reinforce cell wall boundaries to resist infections, or induce localized cell death. A second phenotype, first referred to as induced systemic resistance (ISR), is mediated by jasmonic acid (JA) and/or ethylene, which are produced following applications of some nonpathogenic rhizobacteria. Interestingly, the SA- and JA-dependent defense pathways can be mutually antagonistic, and some bacterial pathogens take advantage of this to overcome the SAR. For example, pathogenic strains of Pseudomonas

Table 3. Bacterial determinants and types of host resistance induced by biocontrol agents

Bacterial strain	Plant species	Bacterial determinant	Type	Reference
Bacillus mycoides strain Bac J	Sugar beet	Peroxidase, chitinase and β-1,3-glucanase	ISR	Bargabus et al. (2002)
Bacillus pumilus 203-6	Sugar beet	Peroxidase, chitinase and β-1,3-glucanase	ISR	Bargabus et al. (2004)
Bacillus subtilis GB03 and IN937a	*Arabidopsis*	2,3-butanediol	ISR	Ryu et al. (2004)
Pseudomonas fluorescens strains				
CHA0	Tobacco	Siderophore	SAR	Maurhofer et al. (1994)
	Arabidopsis	Antibiotics (DAPG)	ISR	Iavicoli et al. (2003)
WCS374	Radish	Lipopolysaccharide	ISR	Leeman et al. (1995)
		Siderophore		Leeman et al. (1995)
		Iron regulated factor		Leeman et al. (1995)
WCS417	Carnation	Lipopolysaccharide	ISR	Van Peer and Schipper (1992)
	Radish	Lipopolysaccharide	ISR	Leeman et al. (1995)
		Iron regulated factor		Leeman et al. (1995)
	Arabidopsis	Lipopolysaccharide	ISR	Van Wees et al. (1997)
	Tomato	Lipopolysaccharide	ISR	Duijff et al. (1997)
Pseudomonas putida strains	*Arabidopsis*	Lipopolysaccharide	ISR	Meziane et al. (2005)
WCS 358	*Arabidopsis*	Lipopolysaccharide	ISR	Meziane et al. (2005)
		Siderophore	ISR	Meziane et al. (2005)
BTP1	Bean	Z,3-hexenal	ISR	Ongena et al. (2004)
Serratia marcescens 90-166	Cucumber	Siderophore	ISR	Press et al. (2001)

Syringae produce coronatine, which is similar to JA, to overcome the SA-mediated pathway. Because the various host-resistance pathways can be activated to varying degrees by different microbes and insect feeding, it is plausible that multiple stimuli are constantly being received and processed by the plant. Thus, the magnitude and duration of host defense induction will likely vary over time. Only if induction can be controlled, i.e. by overwhelming or synergistically interacting with endogenous signals, will host resistance be increased.

A number of strains of root-colonizing microbes have been identified as potential elicitors of plant host defenses. Some biocontrol strains of Pseudomonas sp. and Trichoderma sp. are known to strongly induce plant host defenses. In several instances, inoculations with plant-growth-promoting rhizobacteria (PGPR) were effective in controlling multiple diseases caused by different pathogens, including anthracnose (Colletotrichum lagenarium), angular leaf spot (Pseudomonas syringae pv. lachrymans and bacterial wilt (Erwinia tracheiphila). A number of chemical elicitors of SAR and ISR may be produced by the PGPR strains upon inoculation, including salicylic acid, siderophore, lipopolysaccharides, and 2,3-butanediol, and other volatile substances (Van Loon et al. 1998, Ongena et al. 2004, Ryu et al. 2004). Again, there may be multiple functions to such molecules blurring the lines between direct and indirect antagonisms. More generally, a substantial number of microbial products have been identified as elicitors of host defenses, indicating that host defenses are likely stimulated continually over the course of a plant's lifecycle. Excluding the components directly related to pathogenesis, these inducers include lipopolysaccharides and flagellin from Gram-negative bacteria; cold shock proteins of diverse bacteria; transglutaminase, elicitins, and β-glucans in Oomycetes; invertase in yeast; chitin and ergosterol in all fungi; and xylanase in Trichoderma. These data suggest that plants would detect the composition of their plant-associated microbial communities and respond to changes in the abundance, types, and localization of many different signals. The importance of such interactions is indicated by the fact that further induction of host resistance pathways, by chemical and microbiological inducers, is not always effective at improving plant health or productivity in the field.

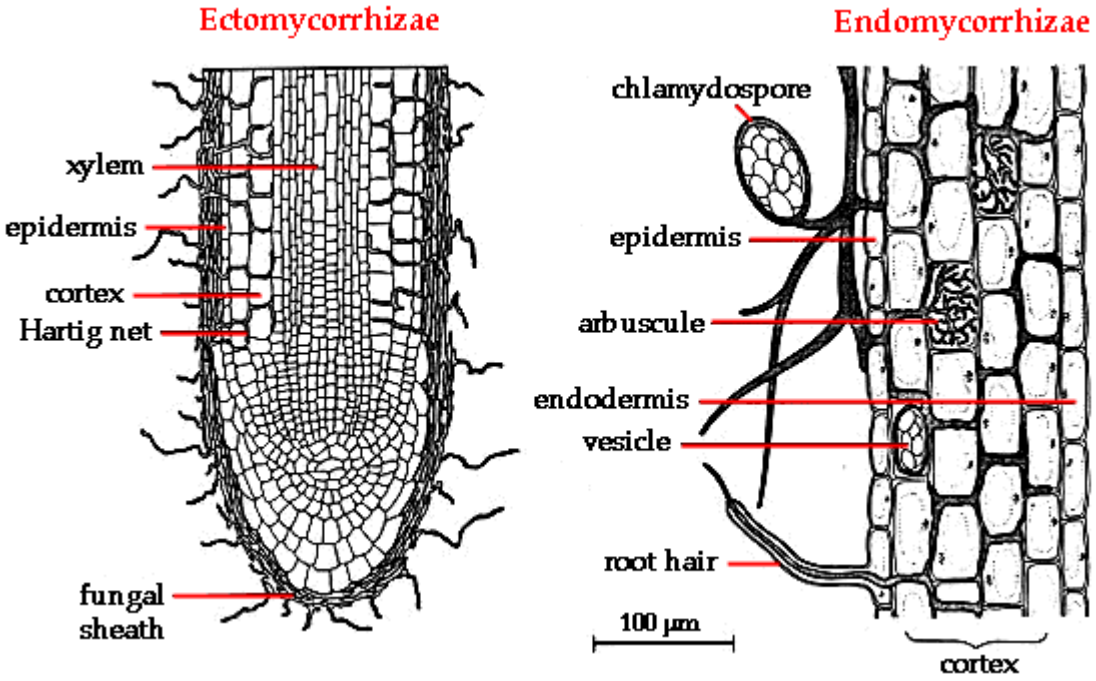
Microbial diversity and disease suppression

Plants are surrounded by diverse types of mesofauna and microbial organisms, some of which can contribute to biological control of plant diseases. Microbes that contribute most to disease control are most likely those that could be classified competitive saprophytes, facultative plant symbionts and facultative hyperparasites. These can generally survive on dead plant material, but they are able to colonize and express biocontrol activities while growing on plant tissues. A few, like avirulent Fusarium oxysporum and binucleate Rhizoctonia-like fungi, are phylogenetically very similar to plant pathogens but lack active virulence determinants for many of the plant hosts from which they can be recovered. Others, like Pythium oligandrum are currently classified as distinct species.

However, most are phylogenetically distinct from pathogens and, most often; they are subspecies variants of the same microbial groups. Due to the ease with which they can be cultured, most biocontrol research has focused on a limited number of bacterial (Bacillus, Burkholderia, Lysobacter, Pantoea, Pseudomonas, and Streptomyces) and fungal (Ampelomyces, Coniothyrium, Dactylella, Gliocladium, Paecilomyces, and Trichoderma) genera. Still, other microbes that are more recalcitrant to in vitro culturing have been intensively studied. These include mycorrhizal fungi, e.g. Pisolithus and Glomus spp. that can limit subsequent infections, and some hyperparasites of plant pathogens, e.g. Pasteuria penetrans which attack root-knot nematodes. Because multiple infections can and do take place in fieldgrown plants, weakly virulent pathogens can contribute to the suppression of more virulent pathogens, via the induction of host defenses. Lastly, there are the many general micro- and meso-fauna predators, such as protists, collembola, mites, nematodes, annelids, and insect larvae whose activities can reduce pathogen biomass, but may also facilitate infection and/or stimulate plant host defenses by virtue of their own herbivorous activities.

While various epiphytes and endophytes may contribute to biological control, the ubiquity of mycorrhizae deserves special consideration. Mycorrhizae are formed as the result of mutualist symbioses between fungi and plants and occur on most plant species. Because they are formed early in the development of the plants, they represent nearly ubiquitous root colonists that assist plants with the uptake of nutrients (especially phosphorus and micronutrients). The vesicular arbuscular mycorrhizal fungi (VAM, also known as arbuscular mycorrhizal or endomycorrhizal fungi) are all members of the zygomycota and the current classification contains one order, the Glomales, encompassing six genera into which 149 species have been classified. Arbuscular mycorrhizae involve aseptate fungi and are named for characteristic structures like arbuscles and vesicles found in the root cortex. Arbuscules start to form by repeated dichotomous branching of fungal hyphae approximately two days after root penetration inside the root cortical cell. Arbuscules are believed to be the site of communication between the host and the fungus. Vesicles are basically hyphal swellings in the root cortex that contain lipids and cytoplasm and act as storage organ of VAM. These structures may present intra- and inter-cellular and can often develop thick walls in older roots. These thick walled structures may function as propagules. During colonization, VAM fungi can prevent root infections by reducing the access sites and stimulating host defense. VAM fungi have been found to reduce the incidence of root-knot nematode. Various mechanisms also allow VAM fungi to increase a plant's stress tolerance. This includes the intricate network of fungal hyphae around the roots which block pathogen infections. Inoculation of apple-tree seedlings with the VAM fungi Glomus fasciculatum and G. macrocarpum suppressed apple replant disease caused by phytotoxic myxomycetes.

VAM fungi protect the host plant against root-infecting pathogenic bacteria. The damage due to Pseudomonas syringae on tomato may be significantly reduced when the plants are well colonized by mycorrhizae. The mechanisms involved in these interactions include physical protection, chemical interactions and indirect effects (Fitter and Garbaye 1994). The other mechanisms employed by VAM fungi to indirectly suppress plant pathogens include enhanced nutrition to plants; morphological changes in the root by increased lignification; changes in the chemical composition of the plant tissues like antifungal chitinase, isoflavonoids, etc. (Morris and Ward 1992); alleviation of abiotic stress and changes in the microbial composition in the mycorrhizosphere . In contrast to VAM fungi, ectomycorrhizae proliferate outside the root surface and form a sheath around the root by the combination of mass of root and hyphae called a mantle. Disease protection by ectomycorrhizal fungi may involve multiple mechanisms including antibiosis, synthesis of fungistatic compounds by plant roots in response to mycorrhizal infection and a physical barrier of the fungal mantle around the plant root (Duchesne 1994). Ectomycorrhizal fungi like Paxillus involutus effectively controlled root rot caused by Fusarium oxysporum and Fusarium moniliforme in red pine. Inoculation of sand pine with Pisolithus tinctorius, another ectomycorrhizal fungus, controlled disease caused by Phytophthora cinnamomi.



With the of a neat and labelled diagram explain VAM (vesicular arbuscular mycorrhizae)

Because plant diseases may be suppressed by the activities of one or more plantassociated microbes, researchers have attempted to characterize the organisms involved in biological control. Historically, this has been done primarily through isolation, characterization, and application of individual organisms. By design, this approach focuses on specific forms of disease suppression. Specific suppression results from the activities of one or just a few microbial antagonists. This type of suppression is thought to be occurring when inoculation of a biocontrol agent results in substantial levels of disease suppressiveness. Its occurrence in natural systems may also occur from time to time. For example, the introduction of Pseudomonas fluorescens that produce the antibiotic 2,4-diacetylphloroglucinol can result in the suppression of various soilborne pathogens . However, specific agents must compete with other soil- and root-associated microbes to survive, propagate, and express their antagonistic potential during those times when the targeted pathogens pose an active threat to plant health. In contrast, general suppression is more frequently invoked to explain the reduced incidence or severity of plant diseases because the activities of multiple organisms can contribute to a reduction in disease pressure. High soil organic matter supports a large and diverse mass of microbes resulting in the availability of fewer ecological niches for which a pathogen competes. The extent of general suppression will vary substantially depending on the quantity and quality of organic matter present in a soil (Hoitink and Boehm 1999). Functional redundancy within different microbial communities allows for rapid depletion of the available soil nutrient pool under a large variety of conditions, before the pathogens can utilize them to proliferate and cause disease. For example, diverse seed-colonizing bacteria can consume nutrients that are released into the soil during germination thereby suppressing pathogen germination and growth . Manipulation of agricultural systems, through additions of composts, green manures and cover crops is aimed at improving endogenous levels of general suppression.

Biocontrol research, development, and adoption

Biological control really developed as an academic discipline during the 1970s and is now a mature science supported in both the public and private sector. Research related to biological control is published in many different scientific journals, particularly those related to plant pathology and entomology. Additionally, three academic journals are specifically devoted to the discipline (i.e. Biological Control, Biocontrol Research and Technology, and BioControl). In the United States, research funds for the discipline are provided primarily by several USDA programs. These include the Section 406 programs, regional IPM grants, Integrated Organic Program, IR-4, and several programs funded as part of the National Research Initiative.

Monies also exist to stimulate the development of commercial ventures through the small business innovation research (SBIR) programs. Such ventures are intended to be conduits for academic research that can be used to develop new companies.

Much has been learned from the biological control research conducted over the past forty years. But, in addition to learning the lessons of the past, biocontrol researchers need to look forward to define new and different questions, the answers to which will help facilitate new biocontrol technologies and applications. Currently, fundamental advances in computing, molecular biology, analytical chemistry, and statistics have led to new research aimed at characterizing the structure and functions of biocontrol agents, pathogens, and host plants at the molecular, cellular, organismal, and ecological levels.. Some of the research questions that will advance our understanding of biological controls and the conditions under which it can be most fruitfully applied are listed in Table 4. Table 4:

Table 4: Some current topics of biocontrol research and development and associated questions:

1. The ecology of plant-associated microbes
   - How are pathogens and their antagonists distributed in the environment?
   - Under what conditions do biocontrol agents exert their suppressive capacities?
   - How do native and introduced populations respond to different management practices?
   - What determines successful colonization and expression of biocontrol traits?
   - What are the components and dynamics of plant host defense induction?

2. Application of current strains/inoculant strategies
   - Can more effective strains or strain variants be found for current applications?
   - Will genetic engineering of microbes and plants be useful for enhancing biocontrol?
   - How can formulations be used to enhance activities of known biocontrol agents?

3. Discovering novel strains and mechanisms of action
   - Can previously uncharacterized microbes act as biological control agents?
   - What other genes and gene products are involved in pathogen suppression?
   - Which novel strain combinations work more effectively than individual agents?
   - Which signal molecules of plant and microbial origin regulate the expression of biocontrol traits by different agents?

4. Practical integration into agricultural systems
   - Which production systems can most benefit from biocontrol for disease management?
   - Which biocontrol strategies best fit with other IPM system components?
   - Can effective biocontrol-cultivar combinations be developed by plant breeders?

Over the past fifty years, academic research has led to the development of a small but vital commercial sector that produces a number of biocontrol products. The current status of commercialization of biological control products has been reviewed recently. As in most industries, funding in the private sector research and development goes through cycles, but seems likely to increase in the years ahead as regulatory and price pressures for agrochemical inputs increase.

Most of the commercial production of biological control agents is handled by relatively small companies, such as Agraquest, BioWorks, Novozymes, Prophyta, Kemira Agro. Occasionally, such companies are absorbed by or act as subsidiaries of multi-billion dollar agrochemical companies, such as Bayer, Monsanto, Syngenta, and Sumitomo. Total revenues of products used for biocontrol of plant diseases represented just a small fraction of the total pesticide market during the first few years of the 21st century with total sales on the order of $10 to 20 million dollars annually. However, significant expansion is expected over the next 10 years due to increasing petroleum prices, the expanded demand for organic food, and increased demand for "safer" pesticides in agriculture, forestry, and urban landscapes.

Growers are interested in reducing dependence on chemical inputs, so biological controls (defined in the narrow sense) can be expected to play an important role in Integrated Pest Management (IPM) systems. A model describing the several steps required for a successful IPM has been developed .In this model, good cultural practices, including appropriate site selection, crop rotations, tillage, fertility and water management, provide the foundation for successful pest management by providing a fertile growing environment for the crop. The use of pest- and disease-resistant cultivars, developed through conventional breeding or genetic engineering, provides the next line of defense. However, such measures are not always sufficient to be productive or economically sustainable. In such cases, the next step would be to deploy biorational controls of insect pests and diseases These include BCAs, introduced as inoculants or amendments, as well as active ingredients directly derived from natural origins and having a low impact on the environment and non-target organisms. If these foundational options are not sufficient to ensure plant health and/or economically sustainable production, then less specific and more harmful synthetic chemical toxins can be used to ensure productivity and profitability. With the growing interest in reducing chemical inputs, companies involved in the manufacturing and marketing of BCAs should experience continued growth. However, stringent quality control measures must be adopted so that farmers get quality products. New, more effective and stable formulations also will need to be developed.

Most pathogens will be susceptible to one or more biocontrol strategies, but practical implementation on a commercial scale has been constrained by a number of factors. Cost, convenience, efficacy, and reliability of biological controls are important considerations, but only in relation to the alternative disease control strategies.

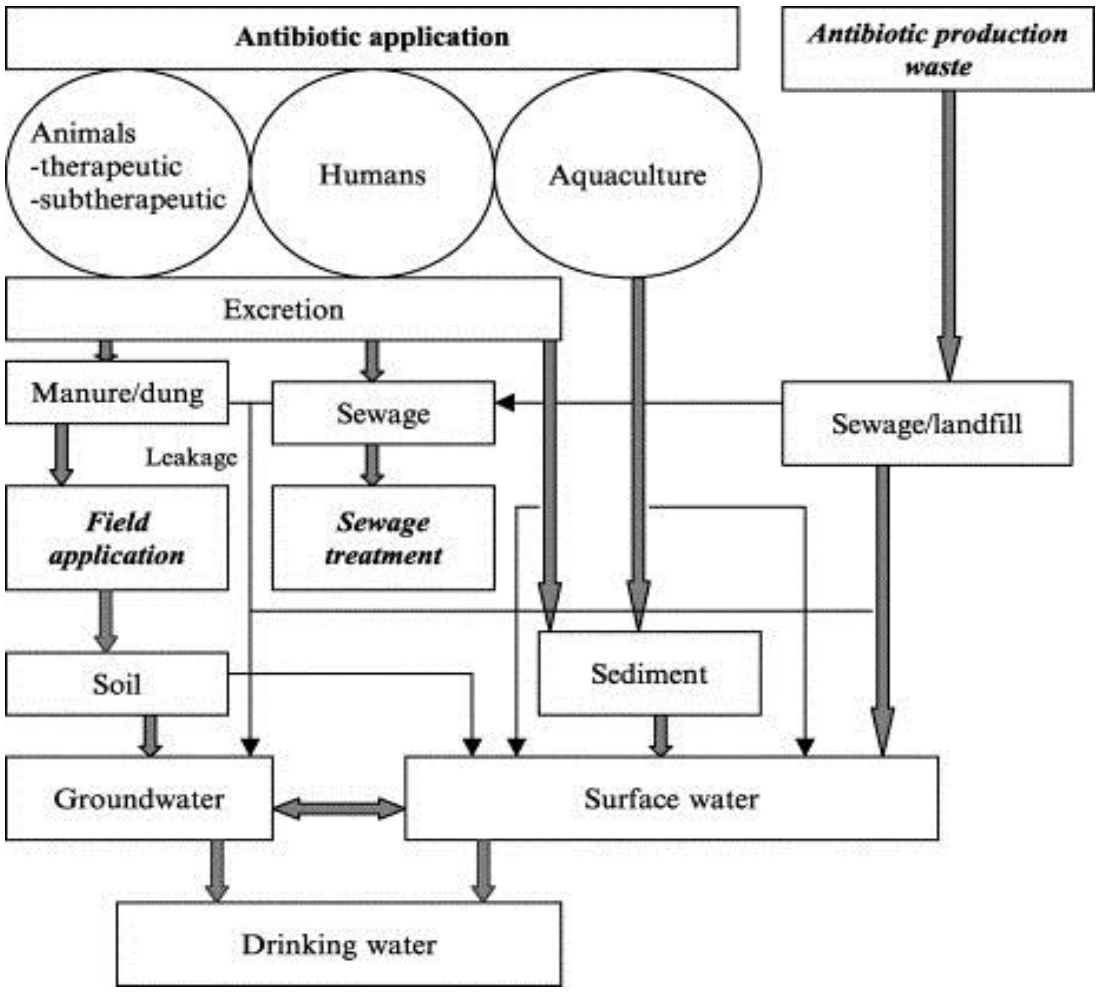
Cultural practices (e.g. good sanitation, soil preparation, and water management) and host resistance can go a long way towards controlling many diseases, so biocontrol should be applied only when such agronomic practices are insufficient for effective disease control. As long as petroleum is cheap and abundant, the cost and convenience of chemical pesticides will be difficult to surpass. However, if the infection court or target pathogen can be effectively colonized using inoculation, the ability of the living organism to reproduce could greatly reduce application costs. In general, though, regulatory and cultural concerns about the health and safety of specific classes of pesticides are the primary economic drivers promoting the adoption of biological control strategies in urban and rural landscapes. Self-perpetuating biological controls (e.g. hypovirulence of the chestnut blight pathogen) are also needed for control of diseases in forested and rangeland ecosystems where high application rates over larger land areas are not economically-feasible. In terms of efficacy and reliability, the greatest successes in biological control have been achieved in situations where environmental conditions are most controlled or predictable and where biocontrol agents can preemptively colonize the infection court. Monocyclic, soilborne and postharvest diseases have been controlled effectively by biological control agents that act as bioprotectants (i.e. preventing infections). Specific applications for high value crops targeting specific diseases (e.g. fireblight, downy mildew, and several nematode diseases) have also been adopted. As research unravels the various conditions needed for successful biocontrol of different diseases, the adoption of BCAs in IPM systems is bound to increase in the years ahead.

Agricultural antibiotics

The term antibiotics encompass a wide range of chemical substances that are produced naturally, semi-synthetically, and synthetically, and are used to inhibit (bacteriostatic) bacterial growth or kill them (bactericidal) . They are categorized based on their effects as either bacteriostatic or bactericidal, and on their series of efficacy, as narrow or broad-spectrum antibiotics. Furthermore, the classes of drugs that are more widely used in agriculture at the global level, which are of growing scientific concern with regards to their potential adverse effects and risk management steps, include the tetracyclines, aminoglycosides, β-lactams, lincosamides, macrolides, pleuromutilins, and sulphonamides . Gelband et al. noted that these antibiotics have the same mode of actions or belong to the same general classes as those used for humans; a situation that demands the judicious use of these drugs in animal farming, as there is bound to be a degree of interaction between animals and humans.

Markedly, the antibiotic consumption patterns in agriculture vary across regions and countries in the developing world, and even antibiotics that have been banned in other countries, including the developed countries, are still being used in most developing countries . However, the antibiotic consumption profiles in developing countries are greatly influenced by the gross abuse and misuse of antibiotics due to their availability over the counter, through unregulated supply chains as well as the purchase without prescriptions . Also, Van Boeckel et al. projected that the antibiotic consumption will approximately double in the BRICS countries consisting of Brazil, Russia, India, China, and South Africa. The forecast is propelled by a shift to large-scale farms requiring the routine use of antibiotics to maintain the health of animals and productivity. The shift is caused by the progress in consumer demand for animal products. Resistance to antibiotics is an inherent side effect associated with the overuse, abuse, or substantial use of antibiotics .

The antibiotic resistance pattern varies between regions and countries corresponding to the degree of antibiotic consumption, which is guided and regulated by the antibiotic policies of a particular country . Nevertheless, China has been registered as the world's leading producer and consumer of both animals and human antibiotics. Antibiotic-related crisis is ascribed to the misuse of antibiotics that are, ultimately, discharged into the environment, the presence of antibiotic residues (parent antibiotic or its metabolites or both found in animal derived products) in livestock products and wastes, and lastly, the lack of stringent and effective supervision and control over antibiotics production, use, and disposal. Human activities in response to industrialization drastically heightened the availability of antibiotic residues in food and the environment, and the development and distribution of antibiotic resistant bacteria along with their resistance genes, thus causing an increase in the abundance of resistant bacteria and genes.



Antibiotic Use in Agriculture and Its Impact on the Terrestrial Environment

The antibiotic residues, and antibiotic-resistant bacteria and resistance genes are considered as environmental pollutants and responsible for a tenacious public health crisis throughout the globe. The health challenges linked to antibiotic-resistant microorganisms are more about restricted therapeutic remedies in most developing countries that lack access to good quality treatment, thus, accentuating infection as an important root of morbidity and mortality. However, the soil and water environment have been regarded as vital reservoirs and sources of antibiotic resistance; more so, as they are affected by agriculture. Not only does the administration of antibiotics in food-producing animals facilitate antibiotic resistance, but it may also result in the presence of antibiotic residues (including the parent compounds or its metabolites, or both) in animal-derived products (muscles, kidney, liver, fat, milk, and egg) available for human consumption.

However, these antibiotic residues have been reported to exert a huge and negative impact on public health and food safety with regards to drug toxicity, immunopathological diseases, carcinogenicity, allergic reactions, and drug sensitization, amongst others. These adverse impacts tend to be influenced by land use, contaminated water sources, national policies (that symbolize production, trade, animal health, and food security), national and international trade, animal demography, and interactions between the human populations as well, as they are reported to vary considerably between regions and countries.

In a nutshell, antibiotic resistance is observed as a "One Health subject", both as a cause and solution encompassing the interactions between humans, animals, and the environment. Accordingly, in an attempt to contain antibiotic resistance, the World Health Organisation instituted a Global Action Plan (GAP) which demands that each country should develop national action plans in line with the key actions of the GAP, but with respect to its financial resources and extent of its problems. Surveillance and monitoring of antibiotic use and antibiotic resistance is one facet of the strategies against antibiotic resistance. However, developing countries encounter challenges regarding surveillance systems because of lack of capacity and integration.

This paper assembles information about antibiotic and antibiotic resistance in animals, animal-derived products, and the agriculture-impacted environment. Basically, it covers antibiotics used in agriculture, ways through which they end up in the environment causing antibiotic pollution, and on the other hand, the consequential effects of antibiotic residues on public health. In depth, the consequential and devastating effect of antibiotic use, known as antibiotic resistance, has been deliberated on to include salient aspects, such as the determination of antibiotic resistance, antibiotic resistance in livestock farming, as well as antibiotic resistance in manure-impacted environment (soil and water).

2. Antibiotics in Agriculture

The use of antibiotics is not only constrained to the clinical settings, as prescriptions involved in the therapeutic regimens for the eradication of diseases in humans. It is also employed in livestock farming, where antibiotics can be used for disease treatment of animals, and in sub-therapeutic levels in concentrated animal feed for growth promotion, improved feed conversion efficiency, and for the prevention of diseases. Of great concern, the uses, types, and mode of actions of the antibiotics employed in agriculture and veterinary practice are closely related or the same (that may belong to the same general classes, function and act in similar ways) to those prescribed to humans.

Clearly, the choice of antibiotics and the antimicrobial consumption pattern demonstrates geographical variation across the continents being influenced by the food animal species, regional production patterns and types of production system, intensive or extensive farming, purpose of farming (commercial or industrial or domestic), lack of clear legislative framework or policies on the use of antibiotics, as well as the size and socioeconomic status of the population, and the farmers in particular.

The inclusion of nonessential antibiotics in animal feed for growth promotion purposes remains largely unregulated in the underdeveloped countries. The persistent use of these nonessential antibiotics in livestock farming can be attributed to the expansion and greater concentration of farmlands, inadequate governmental policies, and control over the use and sales of antibiotics, reduced use of infection control measures, and the unwillingness of farmers to execute delegated changes in farm practices. Developing countries continue to employ the antimicrobial agent for growth promotion to maintain the healthy state of the animals, to increase productivity, and raise incomes for the farmers. However, these are contradictory to the Swedish agricultural data, as it recorded no loss of production after the ban exercise.

Altogether, Boeckel et al. noted that on a global scale, the average antimicrobial agent consumed per annum of animal produced (per kg) varied across the animal species with values of 45 mg/kg, 148 mg/kg, and 172 mg/kg associated with cattle, chicken, and pigs, respectively. Equally, their mode of administration differs with the animal types. In this light, Apata noted that antibiotics were added to water and feed for chicken in sub-therapeutic levels for growth promotion and prophylaxis. This had a devastating effect, as even healthy birds were unnecessarily exposed to antibiotics. Moreover, as these birds compete for food sources, eventually, there exists a difference in the doses consumed between the individuals, with one receiving a higher dose than others. This introduces another differential in the selective pressure on commensals, which could lead to the selection of resistant commensals that would eventually end up in the environment. Singer et al. accorded the administration of antibiotics in animal feed or water, in which the animals are reared in groups, making it difficult to isolate only the infected animals, as well as that the isolation process could be stressful to the animals and dangerous to the veterinarian who has to administer the antibiotic process.

Contrarily, Sekyere, in their study, demonstrated the administration of antibiotics to pigs via the intravenous route for treatment, and in this case, shunned the exposure of healthy animals to antibiotics. However, this mode of administration might cause the accumulation of these drugs in adipose tissues, thereby posing a health risk to consumers of pork fat.

In addition, Cromwell mentioned that varying quantities of antibiotics are being employed at the different stages of livestock production, especially in pig farming, that incorporates four stages viz. gestation, farrowing, weaning, and finishing. Kim et al. emphasized the significant difference in the use of antibiotics amongst piglets, fattening pigs, and sows during therapy and growth promotion; antibiotics are employed in pig farming for treatment, metaphylaxis, prophylaxis, and growth promotion. The authors further recorded a significant difference in the use of antibiotics between the three production systems in poultry farming, including breeding poultry, broilers, and laying hens. Accordingly, these may release different masses of remnant antibiotics into the environment.

Generally, in the developing countries, the level and rate of antibiotic utilization in the farming sector might be influenced by the manner in which the farmers acquire (over the counters) and use these antibiotics (multidrug practices), and also, the presence of existing factors. The existing factors include a high prevalence or level of infections, profound scarcity of state management and development strategies, shortfall in husbandry zone planning, negligible hygienic practices in livestock husbandry in conjunction with the presence of an integrated agricultural system. Specifically, in Vietnam, there has been reported cases of frequent and uncontrolled epidemic diseases, such as the porcine reproductive and respiratory syndrome (PRRS), foot and mouth disease, and digestive tract infections and reproductive disorders in piglets and exotic sows, respectively. The disease conditions necessitate the wide use of antibiotics by producers in livestock for the prevention and therapy of diseases as one of the most likely approaches to combat diseases. Moreover, the country practices an integrated agriculture–aquaculture farming system, whereby the aquaculture is being sustained via livestock and human wastes. This further strengthens the risk of exposure of humans, animals, and environment to available antibiotics.

Similarly, Guetiya Wadoum et al. noted the multidrug practices by farmers in addition to the use of formulations with low doses of antibiotics that do not indicate the active ingredients, or the withdrawal periods in poultry farming, in Cameroon. As a result, they diagnosed the diseases that occurred or threatened the chickens and decided on the types of antibiotics and dosage to employ, as well, the veterinarians even gave wrong diagnosis about the diseases amongst the birds to encourage and promote the sales of their drugs. What a bizarre situation that creates chances for the abuse of antibiotics? Several authors have demonstrated the indiscriminate use of antibiotics by farmers, and attributed it to lack of knowledge on the prudent use of these drugs, and the possible adverse effects associated with their abuse, non-adherence to manufacturer's instructions, and the antibiotic withdrawal periods, unavailability of veterinarians and their services.

Furthermore, inexperienced farmers relied on the knowledge and advice of experienced farmers and local drug sellers for drug administration, and above all, wealthy farmers tended to employ multiple antibiotics, since they have the potential to acquire the drugs. Also, very few animals are referred to the laboratory for diagnosis to identify the causative agent and to assess the antibiotic susceptibility testing prior to antibiotic application.

As a consequence, some of these underdeveloped countries still employ some antibiotics, such as chloramphenicol, tylosin, and TCN (a powder mixture that consisted of oxytetracycline, chloramphenicol, and neomycin) which have been banned for use in the developed countries. Accordingly, these drugs have been associated with aggravation of kidney disease (neomycin), carcinogenicity, mutagenicity, and development of aplastic anemia in humans (chloramphenicol). In addition, Guetiya Wadoum et al. mentioned that TCN and tylosin had to be withdrawn for 21 days and 10 days respectively, before the sales of eggs or meat; a situation which is quite difficult for the farmers to implement and respect. This expedites the consumption, by humans, of poultry products harbouring antibiotic residues.

Notwithstanding, the available limited data on antimicrobial utilization in livestock farming ensues the partial reports of antimicrobial consumption and sales. This is due to the lack of surveillance systems subsidized by the government to monitor antimicrobial use and resistance, the lack of knowledge and the reluctance of food animal producers, animal feed producers, public health and veterinary officers and veterinary pharmaceutical companies to provide such in-depth measurements. In conclusion, the information presented herein is deduced from the findings obtained by several authors who have previously conducted investigations on antibiotic use and antibiotic resistance, yet the rate of antibiotic usage and antibiotic resistance is alarming; imagine the scenario in which the real/actual data has to be presented. Seemingly, there is a need to call for cooperation or team collaboration from individuals, farmers, veterinarians, consumers, and local vendors of pharmaceutical products for the prudent use of antibiotics both in the clinical and agricultural settings across the nations or countries, in a bid to circumvent the rising antibiotic resistance of bacteria.

Antibiotics' Introduction into the Environment

The indiscriminate and abusive use of antibiotics can result in higher concentrations of antibiotics in the environment, which can be termed as antibiotic pollution. The sources via which antibiotics can be released into the environment are diverse, including the human waste streams, and wastes from veterinary use and livestock farming.

Antibiotics used for prophylaxis or therapy in humans contaminate the human waste streams, likewise, the antibiotics used in animals for growth promotion, prevention, and treatment equally contaminate the animals' waste streams. Thus, these are considered as prime sources of antibiotic release into the environment. This is because the administered antibiotics are not fully metabolized, and are released unchanged into the environment, i.e., water, manure or soils. The amount and rate at which the antibiotics are being released into the environments depends on the specific antibiotic and its administered dosage, as well as the species and the age of the animals. Nevertheless, these waste streams will contain both the antibiotics and resistance genes; both considered as pollutants, and their fate in the environment differ.

Furthermore, antibiotics and their metabolites contained in stockpiled animal manure may seep through the pile to surface and groundwater, and also into the soil. This is especially so for antibiotics with high water affinity or which are water soluble, thus making their spread and ecotoxicity in the environment faster, and widely with the aid of water fluidity. In the same view, antibiotics can be introduced into the environment via soil fertilization with raw animal manure, irrigation with wastewater generated from farm activities, or via accidental release by runoffs from farms. Interestingly, Hamscher et al. noted that dust contaminated with antibiotics from farms could equally serve as another route of environmental release of these drugs. Chee-Sanford et al. also emphasized the release of antibiotics into the environment via the dispersal of feed and accidental spill of products, as well as discharges.

In addition, Sekyere noted that pig farmers in some different districts in the Ashanti Region of Ghana do not secure their antibiotics, thereby making them freely accessible for use and abuse by unauthorized persons and children. Also, the farmers disposed of their used antibiotic containers by merely throwing them into drains, refuse dumps, or onto bare ground, instead of burying them as recommended. The author further mentioned that these antibiotics were stored under suboptimal environmental conditions, vulnerable to temperature fluctuations that could accelerate their decomposition, thereby causing a reduction in their concentration and efficacy during administration. Such circumstances promote antibiotic resistance of bacteria living in the gastrointestinal tracts of the animals, due to constant exposure to sublethal levels of these antibiotics, or could even cause prompt administration of an overdose of the antibiotics which is noted to be inefficient. More especially, in commercial and intensive poultry farming, antibiotics may be administered to the entire animal population in feed or water, rather than targeting only the diseased animals. Thus, resistance becomes unavoidable.

Interestingly, antibiotics produced naturally by environmental microorganisms, to deter competitors from living space and food, are gradually accumulating in the environment. Seemingly, antibiotics are released from their production facilities in high concentrations into the environment. Also, Sahoo and colleagues noted that antibiotics could be found in the natural environment via improper disposal of out-of-date drugs from pharmaceutical shops, and unwanted, expired household pharmaceuticals.

Accordingly, these antibiotics released usually consist of different types, and consequently, they do not degrade, all at the same time, i.e., they degrade at different rates in the environment over time by the main elimination processes, including sorption, photo degradation, biodegradation, and oxidation. Albeit, other applied methods, such as adsorption, filtration, coagulation, sedimentation, advanced oxidation processes have been implemented. Specifically, several findings have demonstrated the use of composting, and anaerobic and aerobic digestion to cause the reduction of the antibiotic's level in manure, wastewater, and sludge, but these processes vary in efficiency with the category of the antibiotics, the conditions employed for composting, as well as the type of livestock manure. Nonetheless, the presence of these antibiotics in the environment may create selective pressure resulting in antibiotic resistance and also the removal processes, reduce the concentrations of these antibiotics, allowing time for the exposed bacteria to develop resistance which may be presented as stress adaptation, co-selection, cross-resistance, and cross-protection.

Moreover, the use of antibiotics urges susceptible bacteria to these antibiotics to develop resistance in a bid to survive. In this view, bacteria prevaricate the inhibitory or bactericidal activities of the antibiotics, and execute resistance by either modifying or altering the target sites (ribosomes) for binding by antibiotics, with the help of ribosomal protection proteins which bind to the ribosomes, thereby preventing the binding and interference of protein synthesis or neutralizing antibiotics via enzymes produced by adding acetyl or phosphate groups to the precise site on the antibiotics, or finally, via changing of membrane permeability due to the presence of efflux pumps on the cell membrane. Furthermore, the sensitive bacteria tend to survive in an antibiotic polluted environment by acquiring antibiotic resistance genes from other bacteria or phages (lateral gene transfer), undergo mutations in specific antibiotic gene targets, and by altering of the bacterial surfaces.

Animal-Derived Products and Antibiotic Pollution vs Public Health

In developing countries, food prepared and sold by street vendors is in vogue, and it is still emerging hastily in some countries, notably Indonesia, Cameroon, and Democratic Republic of Congo.

These foods usually comprise of meat (beef, pork, snails) either raw, roasted, or cooked in sauce/stew, starchy foods and snacks, which are sold in restaurants located in public.

Presence of varying concentrations of antibiotic residues in the different animal-derived products in some developing countries. It is for this reason that foodborne outbreaks are highest in developing countries, and dawdles as an issue of public health concern worldwide, because it is indicated as one of the significant food safety hazards concomitant with animal-derived foods. Cooked foods sold on the street have a great socioeconomic impact; they create jobs and provide income to low or unskilled men and women, as well as serve as a major channel for the supply of food to financially handicapped individuals or poor and less privileged individuals. However, there is increased meat consumption to meet the protein demand of the population.

Antibiotics have been reported to accumulate and form residues at varying concentrations in the tissues and organs of food animals, as presented in Table 1. Billah et al. referred to these antibiotic residues as chemical residues or pharmacologically active substances representing either the parent compound or its degraded products, which are released, gathered, or stored in the edible tissues of the animal, due to their use in the prevention, treatment, and control of animal diseases. Undoubtedly, in Cameroon, Guetiya Wadoum et al. demonstrated the presence of chloramphenicol and tetracycline residues in concentrations above the maximum residue limit (MRL) recommended by the European Union in 2010, in edible chicken tissues (muscle, gizzards, heart, liver, kidney) and eggs. Similarly, Billah et al. detected ciprofloxacin in higher concentration in egg white, but in lower concentration in egg yolk during treatment of the birds. Also, Olufemi and Agboola reported a high oxytetracycline residue in edible beef tissues of cattle slaughtered at Akure, in Nigeria, at violating levels beyond the MRL stipulated by WHO. However, of profound concern are circumstances in which diseased animals and animals undergoing therapy could be sold quickly to save funds, or could be slaughtered and used as food or feed for other animals. This causes difficulties in the prophylactic approach to handling epidemic diseases and health risks to consumers, as well as a negative influence on the environment. Van Ryssen reported the use of poultry litter as a feed to farm animals in South Africa, since it is considered as a bulky protein supplement.

Ideally, no animal derived product should be consumed unless there is a complete absence of residual amounts of administered drugs.

Nevertheless, the intriguing fact is that there are constant detectable levels of residues, identified via the help of markedly improved analytical methods. Therefore, the world regulatory authorities have set the MRL for various veterinary drugs that should be expected and considered safe in foods for human consumption. According to Beyene, the diet, age and disease status of the animal added to the absorption, distribution, metabolism, and excretion of the drugs, the extra-label drug use and the improper withdrawal times, amongst others, are the risk factors responsible for the development of residues. In this light, farmers are supposed to adhere and implement the right dosages of the antibiotics, as well as observed their withdrawal periods prior to slaughter and market, in a bid to avoid illegal concentrations of drug residues in the animal products. The withdrawal period (clearance or depletion time) defines the length of time required for an animal to metabolize the administered antibiotics under normal conditions, and also, the time needed for the antibiotic concentration in the tissues to reduce to a safe and acceptable level described as tolerance. It can equally be referred to, the time interval necessary between the last administration of the drug under normal conditions of use to animals and the time when treated animals can be slaughtered to produce foodstuff safe for public consumption. Depending on the drug product, route of administration, and dosage form (even with the same active ingredients), the withdrawal periods vary from a day to several days or weeks, and according to the target animals.

It has been reported that the health of humans correlates directly with the environment (i.e., their habitat and its components, including plants, animals, microorganisms, and other human beings) and the quality of food that they consum. Taking into consideration the growing human population, the changing standard of living conditions, the food shortages, and the greater demands for the intensified production of animal proteins for human consumption across the globe, essential practices to improve on the agricultural and industrial productivity are needed. Of interest is the critical use of antibiotics in agriculture to meet the demands of the rising human population, as the use of antibiotics in this setting has been associated with several benefits. It is therefore anticipated that, in the future, almost all the animals slaughtered and consumed as food must have received a chemotherapeutic or a prophylactic agent of some sort. However, the consumption of these meats, milk, and eggs contaminated with antibiotic residues usually has tremendous impacts on the health of humans. These effects may be direct or indirect, owing to the high dose of the residues, which must have accrued over a prolonged period. They can be exhibited as drug hypersensitivity reactions, aplastic anemia, carcinogenic, mutagenic, immunologic and teratogenic effects, nephropathy, hepatotoxicity, disruption of the normal flora of the intestines, a reproductive disorder, as well as the development of antibiotic-resistant bacteria in the gut.

The Great Challenge: Antibiotics Resistance

The routine employment of antibiotics, for prevention and growth promotion purposes in livestock farming, selects for antibiotic resistance among commensal and pathogenic bacteria. Owing to the fact that most of these antibiotics are not fully metabolized but released into the environment as waste products, antibiotic resistance has an ecological impact, since these waste products still have the potential to influence the bacteria population and promote antibiotic resistance. Cogliani et al. pointed out that the low concentrations of these antibiotics in the environment bring about random and spontaneous mutagenesis. Therefore, the environment has been viewed as a plausible reservoir or pool of antibiotics and antibiotic-resistant bacteria, as well as their resistance genes. It is a situation of great concern to public health facilities worldwide, as bacteria have the capability to transfer resistance genes between strains of the same species and between different species. This is, however, possible due to the fact that the antibiotic resistance genes are located on elements, including transposons, integrons, and plasmids, that can be immobilized. The transmission of these resistance genes is termed horizontal gene transfer (HGT) or lateral gene transfer (LGT), and it does occur via transformation, conjugation, and transduction processes. These processes are responsible for the increasing antibiotic resistance worldwide (because of gene transfers between different bacteria species). LGT has been implicated in the distribution of numerous antimicrobial-resistance determinants, and as the cause of an epidemic in nosocomial and community infections, by conferring resistance to many classes of antimicrobials, which leads to multidrug resistance. Moreover, the employment of broad-spectrum antibiotics creates selective pressure on the bacterial flora, thus increasing the advent of multidrug-resistant bacteria which results in the production of new antibiotic-resistant bacteria with cycles of unpleasant treatments.

(a) *Prevalence of antibiotic resistance in some environmental sources*

Several authors have investigated the prevalence of antibiotic resistance of some bacteria in different environmental samples. These include the following: Ejo et al. in their findings observed an overall prevalence rate of 5.5% of Salmonella isolates identified from raw meat, eggs, milk, and minced meat and burger samples in Ethiopia. The isolates demonstrated relative resistance to ampicillin, tetracycline, and sulphamethoxazole–trimethoprim, with a prevalence of 47.6%. Rasheed et al. equally noted an overall incidence of 14.7% of drug-resistant E. coli obtained from vegetable salad, unpasteurized milk, raw chicken, raw meat, and raw egg surface, with 4% of these isolates exhibiting the extended-spectrum β-lactamase activity.

In addition, Carballo et al. recovered three tetracycline residues and sixty-three antibiotic-resistant Gram-negative bacteria that presented with percentage resistance between 33.3% and 66.7% to five well-known antibiotics employed in livestock farming, viz. tetracycline, chloramphenicol, nalidixic acid, sulphamethoxazole, and ampicillin.

Similarly, Zhu and colleagues, in their findings, noted the high levels of tetracycline concentration in manure and soil samples procured from three large commercial swine farms, from three different regions in China. The authors further revealed a great diversity of antibiotic resistance genes (149 unique ARGs), and emphasized the absolute abundance of 43% of the aminoglycoside phosphorylation gene aphA3 in all the manure samples. In the same country, Gao and co-workers equally unravelled the cefotaxime (CTX)-M gene as the most prevalent extended-spectrum beta-lactamase (ESBL) gene found in E. coli isolates recovered from both pig farm and soil samples. Apparently, Xiao et al. , in a metagenomics analysis of paddy soils from China, provided a broad spectrum profile of antibiotic resistance genes, with multidrug resistance being the most dominant at a level of 38–47.5% of all the samples collected.

Furthermore, Lin et al. isolated and characterized one hundred and thirteen enteric bacteria from the Mhlathuze River, KwaZulu-Natal province, South Africa. Of these bacteria, 75.2% were multidrug resistant, and the enteric isolates obtained from downstream (urban and industrial regions) exhibited greater antibiotic resistance, unlike those from upstream (rural vicinity). This suggests that environmental, industrial, and human activities have a huge impact on the level of environmental antibiotic resistance. Wahome noted the microbial contamination of groundwater samples obtained in Ongata Rongai, Kajiado North County, Kenya, with enteric pathogens including Pseudomonas aeruginosa, Shigella and Vibrio species, E. coli, and Salmonella. The enteric pathogens exhibited high resistance, between 87.5% and 98.5%, to ampicillin, kanamycin, and sulfamethoxazole.

(b) *Methods of determining antibiotic resistance of bacterial isolates*

The antibiotic resistance profile of bacterial isolates to available antibiotics can be determined by using multiple culture-based methods, with the key feature to isolate the target organism through growth on general multipurpose or selective and/or enriched microbiological media, and subsequent evaluation of their growth in response to specific antibiotic concentrations. The culture-based methods offer a link between antibiotic resistance measurement both in the environment and human clinical setting. These cultured-based techniques are designed as susceptibility tests, and the resistance of the bacterium can be deduced directly from the susceptibility testing.

The antibiotic susceptibility test involves both qualitative diffusion and quantitative dilution methods, amongst which is the Kirby–Bauer disc diffusion technique that implements the guidelines adpoted from Clinical Laboratory Standards Institute (CLSI) . In this methodology, the size (diameter) of the zone of inhibition developed around each disc placed on plates of microbiological medium inoculated with the pure culture of bacteria isolate is considered as the degree of sensitivity. The antibiotic susceptibility test with disc diffusion test is, however, regarded as a qualitative test to classify an organism as being susceptible or resistant, and paves the way for the better quantitative tests.

Determining the minimum inhibitory concentration (MIC) of an antibiotic against a bacterial isolate ensures the best quantitative estimate of susceptibility. The MIC describes the lowest or least concentration of a drug that is required to inhibit visible bacterial growth after overnight incubation. The MIC value gives an insight into the degree of resistance, the resistance mechanisms, as well as the resistance genes. It can be determined both by micro broth dilution in microplates, and agar dilution. In between, the E-test (Epsilometer test) combines the diffusion and dilution theories in susceptibility testing, whereby it determines whether the isolate is resistant or susceptible, based on the clear zone of inhibition. At the same time, it quantifies the sensitivity of the isolate by giving a discrete MIC value at the point where the clear zone of inhibition intersects on the test strip that harbours a predefined gradient of continuous antibiotic concentration ranges. Nevertheless, owing to the inherent ability of microbiological agars to detect contamination of inoculum, Jenkins and Schuetz suggested that agar-based methods are more reliable for the detection of antibiotic resistance, unlike the broth dilution methods. In general, both the agar-based and broth dilution methods are faced with challenges, including cost, and that they are time-consuming and labour intensive . It is worth mentioning that the resistance level of a bacterium greatly depends on the type of test and test conditions applied for the determination of resistance, as well as the kind of antibiotics and its mode of action. It is necessary to conduct continuous surveillance of the antibiotic resistance profile of bacteria, since bacteria are vulnerable to develop unpredictable resistance patterns, based on their genetic plasticity. In addition, the susceptibility patterns of a particular bacterium changes with time, geographical location, country, and the prevailing environmental condition. The recovered information gives knowledge and serves as guidelines in antibiotic selection for treatment, to reduce the use of broad-spectrum antibiotics, and also to slow down resistance development, hence predict future resistance in bacterial isolates.

(c) *Zoonoses and agriculture versus clinical setting*

Taking into consideration the routine use of the same antibiotics with similar modes of action both for animal and human purposes, added to the report of zoonoses, which are bacterial infections in humans caused by animal pathogens, including Salmonella spp., Yersinia enterocolitica, Listeria monocytogenes, Staphylococcus spp., Campylobacter jejuni, Enterococcus spp., and Escherichia coli, it is somewhat obvious that antibiotic resistance can be transferred from animals to humans. These bacterial pathogens are of prime importance due to their public health implications, are easily detectable indicator organisms which signify the presence of fecal contamination of the environment, have the potential to acquire resistance genes via lateral gene transfer, and lastly, they can develop resistance to a broad spectrum of last-resort antibiotics. Wegener affirmed that the public health consequences perpetrated by zoonotic pathogens are ever-challenging to evaluate. The consequences involve complex production and distribution systems of food and animals, dissemination of resistance genes and bacterial clones, increased mortality and morbidity, and higher cost in the treatment of disease as well as infections that would have otherwise not have occurred.

These zoonotic pathogens develop resistance in response to the antibiotics used in food animals, and the same strains colonize both animals and humans, and the antibiotic resistance genes can easily spread among the bacterial species or clones that are phylogenetically related . Therefore, these zoonotic bacteria can serve as vectors of antibiotic resistance genes. The resistance can be transferred from animal to animal, or animals to humans, either directly via contact, or indirectly through the food chain, water, sludge-fertilized soils and manure. More specifically, humans can come into contact with antibiotic-resistant bacteria and resistance genes either directly via immediate exposure to animals and biological substances, including urine, feces, milk, semen, and saliva, or indirectly via contact or ingestion of contaminated animal-derived food products. This is, however, the main route for the cause of enteric infections in humans with the zoonotic bacterial pathogens listed above. On the other hand, antibiotic-resistant bacterial strains can be transmitted from humans, including the workers on the farm and their families, to food animals, since it is noted that the digestive tract and the skin of these humans harbour high numbers of commensals, notably *Staphylococcus aureus*. Notwithstanding, the feasibility of transmission is reliant on geographical location, ethnic/cultural practices, religion, hygienic status, farm size, and the type of integrated farming. However, this status quo is true in the rural settings in developing countries, where there is close contact between the animals (specifically, poultry) and humans.

Since farming of indigenous-species animals in small numbers appears to be the most common practice of poultry production systems, in this regard, the animals depend on scavenging as a source of food . Also, in the developing world, biosecurity and food safety measures are inadequate, thus facilitating the direct and indirect mode of acquiring antibiotic-resistant bacterial strains and their resistance genes. Kagambéga et al. isolated and characterized S. typhimurium from poultry and humans that were resistant to the same antibiotics, and harboured the same phage, DT 56, which demonstrated close-relatedness as revealed by pulse field gel electrophoresis.

Lipsitch et al. presented three mechanisms through which antibiotic resistance originating from agriculture can threaten human health, as follows: an individual might be infected by a resistant bacterial pathogen via direct contact or via ingestion of contaminated meat, milk, eggs, or water, and not transmit to other humans. In another scenario, a person might be infected with a resistant pathogen via the aforementioned pathways, with ongoing transmission to other humans and causing infections in some of the individuals. Thirdly, resistance genes derived from the agricultural settings are being introduced into human pathogens by lateral gene transfer. Apart from acquiring resistance by these bacteria, they can equally receive additional virulent genetic elements, leading to an increase in pathogenicity or virulence .

Wegener et al. emphasized that the observed level of antibiotic resistance is closely associated with the amount that is being consumed. Apparently, the level of antibiotic resistance of these bacteria relies on the quantity and the effects of antibiotics in the natural environment originating from antibiotic management within the agricultural and healthcare settings, as well as the antibiotic prescription guidelines. According to Sahoo et al. , the antibiotic prescription is influenced by geographical and climatic factors, socioeconomic conditions, local population density, the behaviour of a particular community towards antibiotic prescription or consumption, and supplier incentives to the prescribers in conjunction to the type of pathogen.

All the same, antibiotic resistance has adverse effects on patients, healthcar systems, and society. More specifically, patients witness a more severe underlying infection, are administered less efficacious but more toxic antibacterial agents, and receive broad-spectrum antibacterial agents, which are so-called reserved or last resorts as an empirical antibiotic regimen. These might result in treatment failure, as well as an increase in the cost of human therapies, due to the severity and persistence of the diseases, added to long hospital stay and prolonged therapy, respectively. Antibiotic resistance equally limits the choice of antibiotics to be implemented in therapy and jeopardizes the chances of the effectiveness of the existing potent antibiotics in treatment regimens used for the eradication of serious but common diseases in the future.

The rising level of antibiotic-resistant bacterial pathogens will eventually hamper future treatment and the prevention of infectious diseases in both animals and humans . Also, the incidence of antibiotic resistance is very critical to the immunocompromised population, since these individuals rely solely on the use of antimicrobials as a defense against pathogens. Unfavourably, the rise in antibiotic resistance presents a potential threat to surgical and advanced therapeutic procedures, including transplantation or anticancer therapy that involve immunosuppression. Therefore, these medical procedures need vigorous anti-infective preventive therapies. However, antibiotic resistance can cause the death of individuals which is the most severe outcome.

(d) Principles of antibiotic use and antibiotic resistance in the clinical and agricultural sectors in both developing and developed countries

The principles or standards established to guide antibiotic use in both agricultural and clinical settings vary between the developed and developing countries, as well as differs from one country to the other. This is visualised from the variation in the antibiotic consumption pattern across the globe, highlighted by Van Boeckel et al. . It is greatly influenced by the antibiotic policies which govern antibiotic use concerning the antibiotic manufacture, antibiotic dispensation, and antibiotic prescription (inappropriate choice and dosing of drugs) of a particular country. However, antibiotic policies are negatively affected by the socioeconomic level (infectious disease burden, income level, educational status, etc.), large population size, and heterogeneity disparity in the healthcare systems in developing countries.

The dearth of functioning antibiotic policies has culminated in the inappropriate use of antibiotics. Van Boeckel et al. stated that about 50% of antimicrobials are used incongruously, regardless of the setting owing to lack of antimicrobial stewardship. Antimicrobial stewardship (formerly called antibiotic policy) entails the choice, dosing, route, and duration of administration of a particular antimicrobial agent. It is defined as the administration of the right drug at the right dose, through the right route, at the right time, to the right patient, to ensure the best clinical outcome for treatment or prevention, thereby causing least harm or toxicity in the patient and future patients. The leading goal of antimicrobial stewardship is to optimize clinical outcomes, to maximize clinical cure or prevention, and to limit the unintended penalties of antibiotic use, including toxicity and the emergence of antibiotic-resistant bacteria.

From the clinical perspective, antibiotics are the most widely used therapeutic agents worldwide. To avoid the irrational and unnecessary use of antibiotics, it is imperative that antimicrobial stewardship is implemented regarding prescriptions guided by appropriate principles based on the patient’s characteristics, the characteristics of the disease-causing agent, and the colonizing microflora.

More specifically, that the pharmacokinetics and pharmacodynamics of the drug, as well as host factors are not left out, in an appropriate antibiotic therapy. The microbiological diagnosis presents as the key procedure in any therapeutic process, where the etiologic agent and the antimicrobial susceptibility patterns, as well as surveillance of the resistance of the pathogen are conducted. Appropriate specimens are collected and submitted to the microbiology laboratory for diagnosis. Diagnosis, in most cases, often relies on culture-based methods which are time-consuming and take several days for a positive result to be obtained. As a consequence, other rapid microbiological methods, including rapid polymerase chain reaction and mass spectrometry have been adopted, and stand a better chance for the future.

In addition, new and rapid molecular tests, including peptide nucleic acid and matrix-assisted laser desorption/ionization technologies have been introduced, that identify common organisms from positive cultures within several minutes. However, especially in critically ill patients (e.g., endocarditis, bacterial meningitis) that are hospitalised, an empirical therapy is decided guided by the clinical presentation of the patients and the site of the infection, in order to reduce the morbidity and mortality rate. Broad-spectrum antibiotics are used that cover a broad spectrum of suspected and non-susceptible pathogens responsible for the clinical presentation. Usually, the combination therapy relies on the synergistic action of the recommended drugs to clear, more rapidly, the infecting microorganism or infection caused by resistant bacteria to multiple antibiotics, or infections caused by more than one organism, as well as to truncate antibiotic therapy . On the other hand, a definitive therapy is employed following the release of the laboratory results on the specific etiologic agent, with the critical opinion to narrow the antibiotic spectrum. Disapprovingly, the definitive therapy is described as the most important component of the antibiotic therapy, as it optimises treatment, reduces costs and toxicity, added to its great possibility of preventing the development of antibiotic resistance. Also, very important at this junction, the clinician consults the modes of action of these drugs, whether they are bacteriostatic or bactericidal, but in more serious infections, bactericidal antibiotics are preferred. In summary, antibiotics maybe consumed in therapies/treatments as first, second, and third line antibiotics. *First line* drugs are administered to patients guided by the clinical presentation and antibiotic susceptibility results, and are based on their broad availability, relatively low cost, and tolerance. However, if a patient fails to respond to the initial drugs or develops intolerance to drugs and/or relapse of infection occurs, other drugs, known as the second line, are added to the treatment. Also, if resistance to the second line drugs is observed, third line drugs are included in the treatment, even though these drugs are associated with higher risk of toxicity and other side effects, unlike the first and second line drugs .

As indicated above, the use of antibiotics in agricultural settings is not only for therapeutic purposes. The employment of antibiotics in different applications in food animals is described as therapeutic use, prophylactic use, and sub-therapeutic use. Ideally, the use of antibiotics in food animals for therapeutic purposes should be accompanied by antibiotic susceptibility testing (AST). Altogether, the results, the age and immune status of the animal, attributes of the drug (pharmacokinetics and pharmacodynamics), and the cost of the drug, are considered in order to decide on the appropriate drug to be used. As in human medicine, the antibiotics during therapy can be categorized into first, second, and third line drugs. However, with respect to the different animal type and practice, the antibiotics vary in the classification, but the principle still remains that the first line drugs are often used in the treatment of most bacterial infections, and the second and third line options are rarely needed.

However, there is an obvious link between antibiotic use and resistance, both on an individual and population level. The high disease burden, poor hygienic and sanitation conditions, limited access to available antibiotics (due to poverty), disparity in healthcare systems and personnels, over-the-counter purchase of drugs, lack of stringent antibiotic policies (that affect the quality and potency of drugs produced), unregulated prescription principles (that lead to self-mediation and prescription by untrained persons), patient expectations, financial incentives to healthcare providers to prescribe antibiotics in developing countries, cause inappropriate use of antibiotics, resulting in antibiotic resistance . Thus, there exist differences in the antibiotic resistance levels between and within countries. For instance, Gebeyehu et al. gave an insight into the inappropriate use of antibiotics in the rural (29.2%) and urban (31.1%) communities in North West Ethiopia, and attributed the practice to younger age, involvement with a job, paucity of knowledge on the use of antibiotic preparations of humans to animals, and dissatisfaction with the healthcare services.

(e) Containment of antibiotic resistance or strategies implemented to maintain appropriate use of antibiotics

Nevertheless, across the globe, countries, states, and regions within the countries have implemented several procedures to regulate and reinforce rational and prudent use of antibiotics in both the clinical and agricultural sectors, in order to contain antibiotic resistance. All these procedures are necessary to conserve the available antibiotics and to maintain their effectiveness. Antimicrobial stewardship occupies a central role in the endeavour to avoid misuse, overuse, or abuse of antibiotics in both settings.

I.Clinical sector

In the clinical sector, the front-end or preprescription, and the back-end or postprescription approach are implemented with different techniques/strategies to optimize the use of antibiotics. These techniques include formulary restrictions, order sets and treatment algorithms, clinical guidelines, education, pharmacodynamic dose optimization, computer-assisted decision support system, pharmacist-driven intravenous to oral switch programs, pharmacy dosing programs, and antibiotic cycling. The back-end approach offers as a better option because it uses prospective review and feedback, and focuses on de-escalation, which permits the modification (a change, adjustment/reduction, discontinuation) of initial empirical antibiotic therapy relying on the culture data, clinical status of the patient, as well as the other laboratory results. The antibiotic de-escalation therapy is the key component within antimicrobial stewardship.

Apparently, McKenzie et al. demonstrated that antibiotic restriction, education of prescribers and patients, and prescription feedbacks as antimicrobial stewardship strategies have improved with the prudent use of antibiotics in Australian hospitals. In addition, the California state in the United States has instituted antimicrobial stewardship in its state legislation. In addition, some countries (e.g., Brazil and Mexico) have implemented policies to regulate and prohibit the sales of over-the-counter antibiotics without prescription. Also, the Central Drugs Standard Control Organization (CDSCO) in India instigated Schedule H1, a stricter regulation, unlike Schedule 1, in a bid to prohibit the sales of over-the- counter antibiotics. According to Laxminarayan and Chaudhury, Schedule 1 harbours antibiotics that must be sold with a valid prescription issued by a registered medical practitioner, and the pharmacist is required to retain a separate register that carries the contact details of the prescribing doctor, the name of the patient, as well as the name and quantity of the drug that is dispensed. The register is kept for three years, and the information contained therein is subject to audit by the government.

Furthermore, the Medicine Control Council (MCC) as part of the National Drug Policy in South Africa subscribes to the World Health Organization Certification scheme. It is mandated to register and relicense, conduct dossier-based medicine evaluation and laboratory-based testing of all medicines utilized in the country in conformity to the criteria for medical evaluation and good manufacturing practice. Moreover, Essential Drug Lists (EDLs) and Standard Treatment Guidelines (STGs) are developed as part of the strategy of National Health policy (NHP) in South Africa, so as to ensure that drugs are readily available and accessible at primary care and at the hospital level, in addition to limiting the choice of antibiotics use via replacement with formularies in the public sector.

Thus, the practice results rational prescribing. Gelband and Duse highlighted that as a regulatory strategy in South Africa, only licensed practitioners might prescribe and/or dispense antibiotics, and the antibiotics are available only on prescription, but not bought over the counter like in other developing countries. In the same way, antibiotic use was substantially decreased at the primary care in Thailand, as well as nationwide actions were demonstrated to address the problem of inappropriate antibiotic use through strengthening of hospital drug and therapeutics committee, engagement in a project based on multifaceted behavioural change intervention, and updating of its essential medicine lists on a regular basis .

Surveillance is the pivot in any control strategy directed against infections in a clinical setting and antimicrobial resistance. Antibiotic surveillance is regarded as the keystone in endorsing antibiotic stewardship, and eases the control of antibiotic resistance. It is also considered as the force behind the programmes geared towards antimicrobial resistance, since it generates reliable and crucial data that can be used to formulate policies on antibiotic use to promote accurate prescriptions of drugs. According to the Global Antibiotic Resistance Partnership—India, real changes in antibiotic consumption or dissemination of resistant bacteria can only be appreciated and/or supported when the resistance level is known and tracked over time, unlike undergoing any type of surveillance. Hence, surveillance of antibiotic resistance complements the surveillance of antibiotic use, and obtained data can be implemented to evaluate the success of intervention programmes. Therefore, salient data required for clinical decision making and national policies can be assembled via surveillance of antibiotic use and antibiotic resistance.

According to Lowmann, the clinical microbiology laboratory undertakes a central role in achieving the key motives of antibiotic stewardship by providing data on culture and susceptibility of the specific patient, and insights for surveillance activities that guides the selection of antibiotics for empirical therapy. Monitoring the consumption of antibiotics is inevitable, as it generates data that can assist in the design and evaluation of interventions aimed at optimizing the use of these antibiotics and prevent rising resistance. The World Health organisation and the Global Action Plan advocated that the quantity and pattern of antibiotics consumed should be monitored as part of surveillance. Pereko and colleagues analyzed prescription claims data and sales data from 2008 to 2011 in the private sector in Namibia to obtain the number of prescriptions that contained antibiotics and the volume of units sold.

The findings highlighted the highest antibiotic consumption by females (53%), followed by individuals of age, 18–45 years (41%) and 34% in Windhoek, with combined therapy of amoxicillin/clavulanic acid as the post prevalent agents used which belong to the family of penicillins.

On the other hand, surveillance of antimicrobial resistance is equally paramount, and should be conducted continuously in order to gain insight into the problem on time, because resistance is evolving. It is defined as the continuous, systematic gathering, analysis, and interpretation of health-related data to monitor and describe an event. Surveillance provides current and salient information needed to develop and monitor antibiotic stewardship programmes, antibiotic formularies, infection control policies, public health interventions, novel antimicrobials, and antibiotic therapy guidelines. It can be executed at the local, regional, national or international level, involving the monitoring of a single bacterial infection or the organism, as well as it can be effected based on funds offered by companies or non-profitable organizations. The procedure provides data on the antibiotic-resistant bacteria, resistance genes, and predicts the rise of antibiotic resistance as depicted from the trend in the antibiotic resistance profiles. Surveillance represents an early-warning system which performs fast distribution of crucial information to public health and regulatory authorities regarding trends and possibility of outbreaks, to bring about timely response measures. Also, Essack reiterated that antibiotic resistance surveillance data are critically needed to update national infection control policies, essential drug lists, and standard treatment guidelines. In addition, Critchley and Karlowsky further emphasized that the data obtained from surveillance and clinical trial can be employed in association to determine breakpoints. Moreover, new medical needs, as well as notorious isolates for the screening of new agents, can be identified from the information contained in surveillance of antibiotic resistance.

In Africa, South Africa is viewed as the only country with the most active surveillance system. The system comprises of two main active groups, namely; the Group for Enteric, Respiratory and Meningeal Disease Surveillance (GERMS) which focuses on obtaining data centered on AIDS-related opportunistic infections, epidemic-prone diseases, and vaccine-preventable diseases. On the other hand, the South African Society for Clinical Microbiology (SASCM) assembles data from the academic hospital on preferred invasive pathogens secluded from the cerebrospinal fluid samples. Aside from these two groups, extra contributions in the collection of data on antibiotic resistance are given by other groups, including the Sexually Transmitted Infection (STI) Reference Centre in alliance with the National Department of Health, the Enteric Diseases Reference United (EDRU) etc. during surveys.

It is worth mentioning that the Gonococcal Antimicrobial Surveillance Programme (GASP) co-ordinated by WHO in Africa was accomplished, owing to the principal role performed by the STI Reference Centre. The STI Reference Centre has provided training and technical assistance in isolate collection and laboratories in Madagascar, Tanzania, Zimbabwe, and Namibia.

Furthermore, in Nigeria, the surveillance system for antibiotic resistance in pulmonary tuberculosis referred to as the National Tuberculosis and Leprosy Control Programme (NTBLCP) is commended by Nasir and co-authors as the only functioning system in which services for drug-resistant TB is provided by four reference laboratories of the country. Also, WHO has introduced the nested PCR equipment in numerous healthcare facilities in Nigeria, therefore, facilitating the molecular-based anti-TB resistance testing regarding rifampicin resistance. Xiao and Li also noted that the Chinese government embarked on a three-year special campaign with the theme "Administrative regulations for the clinical use of antibiotics" to enhance the rational use of antibiotics in 2011. The outcomes of a survey conducted at the tertiary hospitals from 2010 to 2012 involved reductions in antibiotic consumption during prophylaxis in surgical processes (95% to 44.6%), as well as in antibiotic prescriptions to outpatients (22% to 14.7%) and inpatients (68.9% to 54%).

II.Agricultural sector

Antibiotic use for essentially non-medical or non-therapeutic purposes in agricultural settings that are at subtherapeutic levels over an extended period is observed as a major route for the advent of antibiotic resistance and antibiotic-resistant bacteria, and resistance genes have been reported to be transferred to humans . Irrational or non-prudent use of antibiotics in food-producing animals have resulted in antibiotic residues in animal-derived products. Therefore, antimicrobial stewardship is equally implemented to ensure prudent antibiotic use in agriculture, in order to conserve and maintain the effectiveness of available antibiotics, as well as curb the problem of antibiotic resistance and residues in food products derived from animal. Sadly, stewardship interventions in developing countries have often been weakened by the attitude of poorly paid veterinarians who seek supplementary incomes from drug sales, plus the existence of inadequate regulations. Summarily, all stakeholders involved in the fight against antibiotic resistance must address it from the stand point of regulations, surveillance, research, treatment guidelines, infection control, education, and awareness. The National Farmed Animal Health and Welfare Council pointed out that the implementation of antimicrobial stewardship in agriculture can be approached from the following perspectives, including clinical microbiology, infection control (biosecurity), regulations,

surveillance on antibiotic use and resistance, animal management, husbandry, and alternatives to antibiotics. A coordinated network of actions from the veterinarians, livestock producers, pharmacists, veterinary pharmaceutical industries, and regulatory authorities are relevant to enforce prudent antibiotic use.

Following the Global Action Plan on antimicrobial resistance and the Global principles for the containment of antibiotic resistance in animals intended for food presented by WHO, nations are expected to implement measures that are in line with the key actions highlighted for the combat of antimicrobial resistance. Accordingly, Walsh and Wu expressed the interdict on the use of colistin (an antibiotic critical for the treatment of infections caused by highly resistant Gram-negative bacteria in humans) as a feed additive for animals in China. More elaborately, some European countries, including Sweden, Denmark, United Kingdom, Netherlands, etc. introduced bans on the use of antimicrobial growth promoters , while Australia and New Zealand implemented a partial ban. Seemingly, in the United States, the restraint on antimicrobial growth promoters is voluntary. In addition, a tripartite alliance involving WHO, World Organisation of Animal Health (OIE), and Food and Agricultural Organisation of the United Nations (FAO) was formed in 2003, which led to the categorization of veterinary medicines into critically important, highly important, and important drugs for human health. This differentiation is to guide their use in animal agriculture across the globe, hence, combating antimicrobial resistance.

Also, some developing countries do not have national limits for antibiotic residues found in animal-derived food, and thus, rely on international maximum residue limits (MRLs). Therefore, in this continuous fight against antibiotic resistance, the joint FAO/WHO expert committee and Codex Alimentarius Commission updated the MRLs of veterinary drugs in foods of animal origin . Likewise, the Ministry of Health and Family Welfare in India amended the Drugs and Cosmetics regulations, 1945, and set withdrawal limits for drugs used in animal farming. The ministry further emphasized that drug producing companies should equally imprint the withdrawal periods on the containers of drugs meant for animal consumption; however, if not provided, a withdrawal period of not less than 28 days should be considered .

It is worth mentioning that the drug delivery system and the priorities and demands of a country vary from one country to another across the world, thereby causing governments to outline regulations or policies or measures according to their local scheme . Mehrotra et al. found that Health Canada has strengthened the veterinary drug regulatory framework in a bid to enhance antibiotic stewardship.

These were accomplished via increased control over imported veterinary drugs and active pharmaceutical ingredients (API), a compulsory annual compilation of veterinary antibiotic sales by manufacturers, importers, and compounders. Lastly, it created easy access to low-risk veterinary health products (e.g., vitamins, botanicals, traditional medicines, minerals) which serve as alternatives to antibiotics.

Also, antibiotic prescription and administration to farm animals are supervised by veterinarians, and several behavioral studies have proven that the attitude of farmers to antibiotic use is greatly influenced by the veterinarians. Therefore, interventions geared toward the change of prescribing behaviour of the veterinary could go a long way to optimize antibiotic use by farmers. However, the determinant factors, including a personal opinion regarding the contribution of veterinary medicines in antibiotic resistance, professional ethics to alleviate animal suffering, financial dependence on clients, amongst others, have been noted to influence the prescribing behaviour of the veterinarian . Also, Henton et al. emphasized that the registration of over-the-counter drugs for sales is not optional in South Africa, and the drugs are distributed by the manufacturers to veterinary wholesalers, farmers' cooperatives, distributors, and feed mix companies. Also, Lee et al. affirmed that educational programs should be conducted for undergraduate medical and non-medical students in line with generic medicines, mechanism of antibiotic resistance, and prudent use of antibiotics.

Strategies to reduce or limit the therapeutic use of antibiotics in animals via improved animal nutrition, improved living conditions and waste management, biosecurity measures, and improvement in animals' natural immunity can result in infection prevention and control. These strategies, however, will reduce the level and type of antibiotic needed for treatment, because once the animal is exposed to infection, its immune system can fight seriously against the agent, resulting in less severe manifestations . Moreover, reductions in antibiotic consumption can be achieved by using non-antibiotic alternatives, including prebiotics, probiotics, bacteriocins, vaccines, innate immune system potentiators, bacteriophages, and competitive exclusion cultures for non-specific and specific control of enteric pathogens in animals . Nevertheless, guided interventions, such as vaccination, antihelminthic therapy, optimized herd management, improved biosecurity measures, prudent antibiotic use, performed as teamwork involving the farmers and veterinarians, have led to a marked curtail in antibiotic use, especially the critically important antibiotics, with a decrease of 52% and 32% of the pigs from birth to slaughter and breeding animals, respectively .

According to Silbergeld et al. , the largest consumption of antibiotics is found in the production of food animals. The documentation of resistant and multidrug-resistant pathogens in food animals poses threats to public health and food security . Also, the findings of the investigation conducted by Chantziaras et al. revealed a strong correlation between the level of specific antibiotics used and the level of resistance in E. coli strains recovered from pigs, cattle, and poultry of seven European countries. Therefore, data collection of antibiotic use is necessary to understand and counteract antibiotic resistance. The monitoring of antibiotic use is crucial in determining the appropriateness of the drugs in use, detect compliance with prudent drug practice, programmes, or regulations . It can equally contribute in the process of optimizing antibiotic use by identifying the most efficient interventions. In addition, monitoring of antibiotic use creates tolerance in analyzing the temporal trend of antibiotic consumption, and of remarkable interest is the data collected on antibiotic use per animal species. For instance, Schaekel et al. investigated the distribution of antibiotic use per pig age group and active substances (in percentages) used in overall treatment in pig holdings in Germany over a two-year period. The authors found that the types or combination of antibiotics employed varied between the age groups, and the fattening pig experienced a decreasing trend in treatment, but no distinct temporal trend was obtained for the weaners and sucklers. The quantification of antibiotic use in conjunction with data on antibiotic resistance can pave the way for targeted research and development. This is because the data can be used to describe temporal associations between antibiotic use and antibiotic resistance, as well as offer evidence for their links to researchers and policy/decision makers.

Surveillance produces reliable data, and it can be approached from various angles; the antibiotic resistance of pathogens or commensal bacteria, antibiotic use in the treatment of diseases and non-therapeutic uses. The surveillance systems for antibiotic consumption are not standardized and differ from one country to the next. These systems may include; DANMAP (Danish Integrated Antimicrobial Monitoring and Resistance programme, Kongens Lyngby, Denmark), MARAN (Monitoring of Antimicrobial Resistance and Antibiotic usage in Animals in Wageningen, The Netherlands), and SANVAD (South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs, Pretoria, South Africa). Systems designed for antibiotic consumption can be automated systems or randomized field studies, as well as nationwide or cross-sectional or longitudinal studies, which focus on analyzing the sample population of farms.

On the other hand, the cross-sectional studies can be differentiated into national or regional studies. Overall, these studies are heterogenous, and cannot be compared as they consist of differences, including the duration of data collection (yearly, half a year, or even days), different calculation and reporting methods, along with the stratification in the production systems and farm types . Furthermore, some systems monitor and collect data only at the farm level, making it possible for the implementation of benchmarking approaches, whereby farmers or veterinarians are compared and ranked as stated by their level of antibiotic use. By so doing, measures that are relevant to reduce consumption in the top antibiotic users are introduced or implemented .

In addition, Adesokan et al. surveyed the different classes of antimicrobials administered to food animals in South Western Nigeria, and noted that tetracyclines, fluoroquinolones, and followed by beta-lactam/aminoglycosides, were consumed in order of decreasing volume, and mostly in rainy season than dry season. In addition, World Organisation for Animal Health highlighted that 94% of African Countries lack an official surveillance system on the use of antimicrobials in animals . However, though the survey of antimicrobial use in developing countries is inadequate, it demonstrates a high level of farmer prescription, purchase of over-the-counter antibiotics, non-compliance to antibiotic withdrawal periods, and drug counterfeiting.

Surveillance of antibiotic resistance is a vital tool for microbial risk assessment, and the data generated enlighten the understanding of resistance epidemiology, as well as calls for prompt, effective, and early control actions from the government, and also monitor and evaluate the effectiveness of policies implemented by the government . According to Dar et al. , and Gelband and Duse , only a few national, cross-sectional studies on antibiotic resistance of animal-recovered isolates have been carried out in low- and middle-income countries and South Africa, respectively. However, the surveillance systems differ between countries and/or agencies, due to variation in agricultural practices, monitoring needs, and available guidelines . Furthermore, an integrated surveillance of drug-resistant bacteria obtained from humans, food-producing animals, food and other environmental sites, synchronized with the monitoring system for antibiotic use, can assist in the containment of antibiotic resistance. This is described as the “One Health Approach” intended to attain optimal health for humans, domesticated animals, wildlife, plants, and our environment . The first ever integrated animal–human surveillance system was created in Denmark, known as DANMAP (Danish Integrated Antimicrobial Monitoring and Resistance Programme) which addresses the problem of antibiotic resistance with bacteria of food, animal, and human origin .

III.Challenges Faced by Developing Countries Regarding Surveillance Systems

A satisfactory and comprehensive survey is needed to understand the epidemiology of antibiotic resistance in both animals and humans. Even though the global action plan by WHO/OIE/FAO to contain antimicrobial resistance requires each nation or country to implement national action plans, developing countries are still to develop a sustainable surveillance system of antibiotic use and antibiotic resistance. The challenges regarding the surveillance system include:

The countries have just a few laboratories with the potential to conduct quality-assured microbiology and drug sensitivity testing. Årdal et al. strongly affirmed that collection and reporting of data and the strengthening of laboratory capacity are the two related issues in surveillance.

Due to the high burden of infectious diseases and low socioeconomic status of these countries, there is a lack of available resources. According to Nasir et al. , the developing countries lack the funds to purchase reagents and consumables essential for testing antibiotic resistance, thus lack necessary plans for the surveillance of antibiotic-resistant bacteria. The cost necessary for the adequate surveillance, together with the small margin profits in the veterinary sector presents a financial drawback to support surveillance in the veterinary and agricultural sector.

There is a discrepancy in the selection of isolates. Most of the isolates are from clinical cases that are sick individuals (human or animals). Therefore, the sample of isolates is biased toward a more resistant isolate, owing to the previous antibiotic therapy administered. Also, only a few isolates are involved, since the veterinarian decides on the individual animals to refer to the laboratory. Consequently, the proportion of the isolates is not a representative of the bacteria strains under survey taken from animals.

Also, relatively few studies have been conducted on animal-recovered isolates, as well as the criteria for testing isolates differ between countries, likewise, the antibiotics that are tested .

Either at the regional, national, and local levels, there exists variation in obtaining data, owing to differences in laboratory protocols, conditions employed for testing, personnel conducting the drug sensitivity assay, antibiotic policies, quality control and assurance of the laboratory, and considerations regarding breakpoints. In reality, there are no well-known breakpoints for the animal, which results in the adoption of breakpoints values from human medicines. Nevertheless, the standard protocols and breakpoints from the Clinical Laboratory Standard Institute (CLSI) have been adopted by most countries.

These countries also lack stringent and comprehensive policies and plans to circumvent antibiotic resistance. They lack enforcement of regulations regarding prudent antibiotic use, since many are still faced with the problem of purchasing drugs over the counter or without a prescription , and the presence of counterfeit drugs . Nevertheless, even if some data are collected, they fail to translate the surveillance data into policy, especially in South Africa .

At the national level, there is a lack of collaborative measures between the different laboratories regarding surveillance of antibiotic resistance, which might hamper efforts to track emerging resistance and also limit the chances of systematic comparison and evaluation of national activities directed toward the containment of antibiotic resistance.

Antibiotic Resistance in Livestock Farming

According to Woolhouse et al. , antibiotic resistance in livestock farming can be looked at from four different viewpoints, i.e., the animals (cattle, pigs, poultry, sheep) and animal-derived products, farm workers, and farm environmental sites (water, soil, feeds, wastewater, sewage, lagoon, manure, and sludge after treatment). All these constitute the several compartments and different niches in the farm described as an ecosystem . Undoubtedly, farm animals are a very important component in understanding the interplay between humans, animals, and the environment regarding bacteria, antibiotics, and antibiotic resistance gene movement . The digestive tract of animals, like humans (farm workers), is colonized with diverse microorganisms, including commensals and resistant bacteria. Thus, it serves as the most important reservoir of microorganisms. Therefore, it can play a vital role in the dissemination and acquisition of resistant bacteria and their resistance genes.

Shobrak and Abo-Amer noted the occurrence of multidrug-resistant E. coli and E. vulneris in cloacal samples of both migrating and non-migrating birds which served as carriers. Through the release of their fecal residues into water bodies and other environmental sources, it could enable the spread of these resistant bacterial strains and their resistance determinants even to remote areas by means of migration. The anatomical feature of the gut varies between poultry and the other mammalian animals, which in turn influences the intestinal microbiome. The intestinal microbiome changes with age, the type of diet fed, antibiotics ingested, infection with pathogens, amongst other life events . It has been reported that microorganisms in the gut interact extensively with the host, diet, and the intestinal gut microbes, and exert a huge impact on the animal’s immunity and physiology, and ultimately affects the health of the animal and its production .

The continuous antibiotic exposure to animals via oral administration creates selective pressure for the development of resistance, and resistant bacteria associated with animals can then enter into the food chain through the consumption of meat (contaminated during slaughter or processing of carcass, if the gut is accidentally cut or intestine empties its contents into the thoracic and abdominal cavities when the carcass of poultry is gutted during processing) or other animal-derived products, through farm runoff water and other means. However, the greater the quantity of antibiotic used, the higher the selective pressure. Van et al. reiterated that food contaminated with antibiotic-resistant bacteria could cause amplification of resistance genes and facilitate the transfer of the antibiotic resistance determinants to other bacteria of clinical importance found in humans, and can be further transferred within humans to more pathogenic bacteria. Therefore, food or animal-derived products, including meat, milk, and eggs, may represent an active and key medium through which antibiotic resistance determinants are continually being transferred between bacteria, and from animals to humans . Bosco et al. clearly documented the multidrug resistance of Salmonella isolates recovered from cattle, pigs, chickens, eggs, and animal-derived products, as well as cross-species transmission of plasmids between animal and humans in Uganda.

Also, the farm environment is composed of environmental sites, such as manure, wastewater, soils, effluent, and sewage, which serve as hotspots for antibiotic resistance pollution. More specifically, Bester and Essack indicated that animals are very exposed at a high degree to their environment, making it easier for them to be infected with bacteria harboring problematic genetic material, especially from the soil environment. In the same light, animal urine and feces containing antibiotic residues, antibiotic-resistant bacteria, and resistance genes may be released into manure, and animals might, in turn, be allowed to graze on pasture grown on soil fertilized with this raw manure. This, therefore, creates the likelihood of bringing back these xenobiotics to animals and humans.

Nevertheless, manure, which has been described as a hotspot for antibiotic resistance bacteria and antibiotic resistance genes, can serve as a plausible route of transmission of these resistant bacteria and their genes into the soil and water via deliberate or accidental processes . Therefore, it is advised that manure should be treated before land application via biological methods, including anaerobic digestion. However, Resende et al. in their findings noted the prevalence and persistence of potentially pathogenic bacteria which demonstrated multidrug resistance against oxacillin, ampicillin, and levofloxacin, amongst other antibiotics, both in the influent (cattle manure) and effluent (digestate) released from an anaerobic biodigester.

The authors suggested that the rate of survival of these bacteria depended on the temperature of the operating process in association with the duration of the fermentation process and the microbial composition. Ostensibly, Maynaud et al. further confirmed the occurrence of enteric pathogenic bacteria in the digestate obtained from the anaerobic biodigester. The authors emphasized the potential of the viable, but non-cultural state of bacteria, which might cause the regrowth of pathogens during digestate storage, prior to land spreading. Consequently, the need of post treatment of digestate via mechanical, chemical, physical, and biological methods is very vital . In addition, the sanitary risk and microbiological safety of digestate should be evaluated before land application of digestate, in a bid to dodge the ecological, human, animal, and environmental health implications. Nevertheless, it is a call for concern when pathogenic bacteria are present alongside with antibiotic-resistant bacteria in untreated or treated animal manure.

Alternatively, the very common and economic approach to manage manure generated from livestock farming is by application on nearby agricultural fields. However, raw manure can be flushed by heavy rainfall or runoffs from the surface of manure-amended soils into nearby water bodies used by humans for sanitation and domestic purposes . Moreover, due to the constraints on available freshwater resources in developing countries, wastewater serves as a vital source of water and nutrients for irrigation of agricultural fields, in a bid to circumvent the problem of food insecurity in these countries. However, wastewater may infiltrate into groundwater, causing pollution and contamination with toxic chemicals, antibiotics, and organic matter. In addition, owing to the lack of or inefficient wastewater treatment plants, wastewater or improperly treated wastewater is mostly released into surface water bodies that act as reservoirs for domestic and industrial wastes, causing pollution.

Antibiotic Resistance in the Soil Environment

The soil is an ecosystem and a natural resource with unique biodiversity, taking into consideration abundance, quantities of species, and functions of organisms. With respect to total biomass, microorganisms are considered as the principal part of the soil community, and are basically responsible for decomposition of organic matter, degradation of toxic compounds, and nutrient transformation . Interestingly, the soil is composed of microorganisms that produce antibiotic by so doing; it can equally serve as a reservoir of antibiotic-resistant bacteria and resistance genes. The soil serves as a hub to establish connections between the air, water, rocks, and organisms, and it is involved in many different functions termed ecosystem services in the natural world.

Thus, it can be described as quite a large reservoir of antibiotic resistance determinants, since it includes the antibiotic resistance determinants found in all plants, fungi, soil bacteria, small animals, and protists. In addition, Reisenfeld et al. indicated that uncultured soil bacteria are a possible reservoir of antibiotic resistance genes with greater diversity as compared to previous findings, and such diversity can be ascertained and fathomed using culture-independent methods. Furthermore, vegetables grown in unfertilized soil were equally shown to harbour antibiotic-resistant bacteria and resistance determinants that naturally occur in soils .

It is somewhat obvious that the abundance and the mobility of antibiotic-resistant bacteria and resistance determinants in the soil can be greatly influenced by the application of manure (containing antibiotic residues, antibiotic-resistant bacteria, and their resistance genes on mobile elements) during fertilization of the soil, the use of wastewater (black or grey water) for the irrigation of agricultural lands, and the use of antibiotics to treat crop diseases . More specifically, when soils are treated or amended, antibiotics and their degraded metabolites, as well as antibiotic-resistant bacteria and their corresponding resistance genes, are introduced into the soil environment. Therefore, transfer of antibiotic resistance genes becomes inevitably fast, owing to the rapid growth of bacteria and HGT . However, the persistence and the rate of dissipation of the antibiotic resistance genes is dependent on the VGT/HGT of ARG, the transport and viability of the bacteria harbouring the genes, whether the free DNA obtained from cell lysis will be degraded, adsorbed to soil or organic matter, or acquired by new cells, as well as the transportation of the extracellular ARG . However, the introduction into the soil environment via manure fertilization has been reported to cause an alteration in phylogenetic structure, amplification in resistance level, and disturbance in ecological function (e.g., nutrient cycling) in the microenvironment .

Also, the degree or extent of the changes impacted on the soil will depend on the type of manure. In details, varying quantities of antibiotics are employed in livestock, and the quantity depends on the species of animals and the kind of farming system. The level of antibiotics found in manure available for a land application depends on manure management practices, which equally vary depending on the size of the herd, the type of livestock and farm operations, as well as the production stage of the animals . According to Chee-Sanford et al. , the introduction of antibiotics in the soil may give a selective advantage to commensal bacteria harbouring resistance genes, or may create selective pressure, necessitating the acquisition of resistance genes in the commensal population.

Numerous authors have characterized antibiotic-resistant bacteria and resistance genes from vegetables, including lettuce, cabbage, radish, green corn, onion, carrot, and fluted pumpkins grown on manure fertilized soils and irrigated with wastewater . Specifically, Bonyadian et al. , in a study in Iran, demonstrated the presence of antibiotic-resistant verotoxigenic E. coli on vegetable samples collected randomly from retail shops. However, Kumar et al. indicated that these vegetables absorb antibiotic contained in the manure. Therefore, the direct consumption by humans (uncooked or raw) of vegetables might lead to the transfer of antibiotic-resistant bacteria and their resistance genes, which may cause bacterial infections. Notwithstanding, Beuchat and Johannessen et al. suggested that the contamination of vegetable leaves may arise from diverse sources, such as manure, soil, water (for irrigation and cleaning), the handling equipment for product harvesting and processing, the mode of transportation, in addition to animals at both the pre- and post-harvest process.

Antibiotic Resistance in the Water Environment

Microorganisms (specifically, bacteria) do not live in isolation, but are found in milieu/medium (humans, air, water, plants, and soil) known as their habitat (aquatic ecosystem), which offers them with the appropriate nutritional and growth requirements necessary for survival. Consequently, water represents one of the most important habitats for bacteria on the planet earth, and serves as a main natural route for the dissemination of microorganisms between different environmental compartments and/or aquatic ecosystems, humans, and other animals . According to Taylor et al. , the aquatic environment is considered as a fundamental setting for environmental release, transformation, mixing, and persistence of antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes. The water (aquatic) environment can be subdivided into marine and fresh water based on salinity, average temperature, depth, and nutrient content . More elaborately, the microbial aquatic environment includes surface and ground waters, drinking water, tap water, and wastewater. These waters have dynamic and distinct bacterial composition patterns influenced by temporal and spatial unevenness in physicochemical and biotic factors, including environmental stresses and nutrient composition. Nevertheless, some known waterborne bacteria include E. coli, Vibrio, Shigella, and Salmonella species. The aquatic environment has been reported to be the origin and reservoir of antibiotic-resistant bacteria and resistance genes.

Microbial communities respond to drastic changes in the ecosystem functioning, species composition and abundance, due to pollution, resulting in hypoxia, eutrophication, bioaccumulation and dissemination of pathogens.

Consequently, the abundance of antibiotic residues and pool of antibiotic-resistant bacteria and their resistance genes in aquatic ecosystems can be influenced or altered or amplified by the discharge of wastewaters from industrial and municipal wastewater treatment plants, runoff from manure-fertilized agricultural land, leakage from septic tanks and broken sewage pipes, and feces from wildlife, into water bodies .

Furthermore, the aquatic environments offer ecological and economic benefits . Rivers serve as predominant sources of renewable water for freshwater ecosystems and humans, wherein they perform functions such as in irrigation, and are used as drinking water and for recreation . Water is indispensable to life. Therefore, humans need clean and safe drinking water to protect their health from waterborne bacterial infections. Water safe for human consumption needs to be free of pathogenic bacteria, although, the existence of pathogenic bacteria in water is intermittent, changeable, and at low levels, coupled with the fact that the isolation and cultivation of pathogenic bacteria from water sources are not straightforward. Therefore, routine microbiological water analysis does not involve the detection of pathogenic bacteria. Interestingly, Enterococci and E. coli have been used in monitoring the fecal contamination of drinking and recreational water. Nevertheless, fecal pollution of water sources is an increasing problem both in the developed and developing countries. In the latter countries, there may be an increase in the occurrence of pathogenic bacteria in river water near large urban populations, due to inept sewage treatment, fast-growing population, low income, and severe water stress. Hence, infection rates tend to be high, with waterborne pathogens. Mulamattathil et al. recovered coliforms and heterotrophic bacteria, as well as antibiotic-resistant Aeromonas and Pseudomonas species from surface and drinking water in Mafikeng, South Africa. The occurrence of these pathogens in drinking water might indicate serious health implications, especially among the immunocompromised individuals.

(a) Recreational waters and antibiotic resistance

Recreation has a pertinent role in the life of the human population, who always tend to couple the scene with water, be it man-made or natural. It has been reported that antibiotic resistant bacteria can equally be transmitted to humans during recreational use of fresh or marine water, which are natural aquatic habitats. The natural aquatic environment, e.g., the surface waters, such as rivers and sea, serve as a receiving body of runoff water from farmlands fertilized with sewage sludge and animal manure, and wastewater discharged from treatment plants, which contain obvious concentrations of antibiotic resistant bacteria and their resistance determinants, along with biologically active metabolites of antibiotics or the parent compounds. This causes great

environmental damage and eutrophication, which might result in serious degradation of the water quality owing to the influx of high nutrients. The quality of recreational water can be evaluated using fecal indicator bacteria, including E. coli and Enterococcus. Restricted to these two bacteria, the prevalence of antibiotic-resistant bacteria in the natural aquatic environment is always underestimated, because all the studies are geared towards measuring antibiotic resistance in the aforementioned culturable bacteria. However, other bacteria are present in the natural habitat, and might harbour antibiotic resistance genes which may equally contribute in the pool of resistance genes found in the habitat, thus, have the potential to disseminate bacterial resistance . This is affirmed by the findings of Lihan et al. , who through culture-based methods and DNA fingerprinting unraveled the presence of single and multiple antibiotic-resistant Enterobacter, Serratia, Pantoea, Klebsiella, and Citrobacter in water samples obtained from a recreational river located in a resort community in Malaysia Borneo. Besides, the marine settings are special areas desired for recreational purposes and resting, and as such, are exposed to waste from residences, ships, and industries, as well as coastal areas which are site for highly populated cities, extensive fish farming, and recreational areas . The transmission of the resistant bacteria and the resistance genes to humans depends on the type of water sport undertaken, as well as the density of the antibiotic-resistant bacteria in the water. The concentration/magnitude of these resistant bacteria in the recreational waters is determined by the local landscape (e.g., coastal waters), wind speed, ultraviolet radiation, temperature, rainfall, and the source and level of pollution. In a study conducted by Overbey and colleagues, higher levels of E. coli and Enterococcus were enumerated in recreational water samples collected from sewage-impacted sites in five beaches in Galápagos Island, Ecuador. In addition, Fernandes Cardoso de Oliveira et al. revealed a higher level of multiple antibiotic resistance in heterotrophic marine bacteria recovered from sea water and sand in Gonzaguinha, the most organic polluted recreational beach, amongst Ilha Porchat and Guaraú beaches located in Southeast Brazil.

Also, Alipour et al. noted the presence of multiple antibiotic-resistant Enterococcus species characterized from the river and coastal water samples in the Northern part of Iran, therefore, these habitats are not suitable for swimming, as they would increase the risk of human exposure to antibiotic-resistant bacteria and resistance genes. Furthermore, Akanbi et al. demonstrated the multiple antibiotic resistance patterns of Staphylococcus aureus isolated from recreational waters and beach sand identified by the occurrence of mec A, fem A, rpo B, bla Z, erm B, and tet M genes, in the Eastern Cape Province of South Africa.

The beach water and sand can act as a plausible pool for antibiotic-resistant bacteria and resistance genes, and hence facilitate transmission via the direct ingestion of the seawater and/or direct contact with sea water during recreational activities undertaken in these waters. In recent times, the awareness and involvement of many people in water sports have greatly increased, due to an increase in the local population and income earners, as well as tourism.

(b) Drinking water and antibiotic resistance

Most of the population in rural settings and urban settlements rely on untreated groundwater as a source of drinking water, due to the scarcity of fresh surface waters. Untreated groundwater is considered safe for drinking, because it originates from the ground. Therefore, it is described as a natural water habitat protected from human intervention. Apparently, its microbiota reflects the natural population of the habitat. Nevertheless, depending on the location/environment, groundwater becomes vulnerable to contamination with antibiotic residues, antibiotic resistant bacteria, and resistance genes from surface runoff of animal feces deposited on the ground in concentrated animal feeding operations, seepage of liquid/solid manure from storage sites (lagoons), leachate from landfill sites, spillage from broken sewage pipes, and leakage from septic tanks. Also, in the North West province of South Africa, a study conducted by Carstenrevealed a very poor physicochemical and microbiological quality of water samples obtained from boreholes in the Mooi River and Harts River catchment areas. The authors attributed the results to the fecal contamination of groundwater with fecal bacteria and opportunistic bacteria that displayed different levels of antibiotic resistance.

Surprisingly, Kümmerer reported that antibacterial agents found in groundwater are of considerable low concentration. The microbial concentration in groundwater is closely associated with its depth, season, weather conditions, and the type of adjacent land used in the concentrated animal feeding operations.

Wastewater constitutes “black water and grey water”. It describes all water with adversely affected quality due to influences from human activities. Wastewater embodies water from livestock and poultry farms, aquaculture farms, and municipality that is sometimes released into the environment without treatment or used for irrigation by farmers involved in urban agriculture, since it contains significant amounts of micronutrients and organic matter, and provides water. Due to the unavailability of good quality water worldwide, farmers resort to employing wastewater for irrigation, thereby promoting plant growth, water conservation, nutrient recycling, and reductions in inorganic fertilizer applied to the soil, and polluted water being discharged into the surroundings .

Owing to its numerous sources of water collection, wastewater contains diverse elements, inorganic mineral, antibiotic residues, antibiotic-resistant bacteria and resistance genes, human and animal feces and urine, etc. . The sewage or wastewater treatment plant receives the wastewater, and partially treats or treats it, before discharge of its effluents into the environment. Therefore, the wastewater treatment plant serves as a link between human activities and the environment, and serves as a potential reservoir and release channel of antibiotic-resistant bacteria and resistance genes, giving a perfect opportunity for the transfer of antibiotic resistance genes . The biological processes at the wastewater treatment plant might cause a reduction in the volume of antibiotics to varying degrees. However, across the different regions of the world utilizing distinct types of wastewater treatment, these plants have been reported to be responsible for the discharge of about one billion cultivable coliform bacteria into the environment, indicating their inefficiency to reduce antibiotic-resistant bacteria and their resistance genes. Clearly, Adefisoye and Okoh demonstrated the occurrence of multidrug-resistant E. coli from the final discharged effluents of two wastewater treatment plants situated in the Eastern Cape Province, South Africa.

(c) Waterborne disease outbreaks caused by recreational/drinking waters versus antibiotic resistance

Outbreaks of diseases implicating water as a vehicle of transmission are termed waterborne disease outbreaks, because two or more persons are affected by a similar illness after being exposed to water, and are epidemiologically linked by time and by location of the water . Humans become vulnerable to infections when exposed to the beaches when the waters are turbid and experiencing high waves, as well as after heavy rainfall; these cause the waters to be contaminated and unsafe . Depending on the environmental conditions (climactic factors) and the characteristics of the pathogens, the waterborne pathogen grows in the aquatic habitat, however, its number, type, infectivity, and virulence is greatly influenced by the temperature, UV radiation, precipitation patterns, and water availability in the recreational waters .

According to Schets et al., the waterborne illnesses in humans associated with the use of untreated or contaminated recreational water mainly comprise of gastroenteritis and skin infections. The authors further revealed that the bathing sites were predisposed to fecal contamination and environmental conditions that favoured the growth of naturally occurring pathogens. Children are viewed to be at a higher risk of gastroenteritis from recreational waters, because they last longer and swallow more water during swimming, as well as they play in shallow water and sand which are most contaminated, and experience a high tendency of hand to mouth exposure.

However, waterborne infections associated with exposure to drinking water include acute gastroenteritis, typhoid, diarrhoea, acute respiratory and neurological illness, and skin infections . Between 2000 and 2001, South Africa witnessed a surprising and the biggest cholera outbreak in Africa, which involved 114,000 cases across nine provinces and caused 260 deaths, although it originated from KwaZulu Natal province . Equally, Allam et al. reported an outbreak of cholera involving 218 persons, which attacked a greater number of children (aged 5–14 years), males, and individuals greater than or equal to 60 years in Andhra Pradesh, India, and was caused by the contamination of reservoir water with Vibrio cholerae. This outbreak claimed three human lives. Even Biswas and colleagues investigated an outbreak of cholera in Haibatpur village, India, caused by pond water contaminated with Vibrio cholerae. Ramurmutty and Sharma suggested that the recurrent outbreaks of cholera in India are due to the presence of short-term carriers of Vibro cholerae in the communities, the constant change in biotypes and serotypes of this strain, development of resistance to the previous multiple antimicrobial agents used in the treatment of cholera- as well as poor water quality, unhygienic sanitation- and overcrowding.

A shigellosis outbreak among 533 students in a rural elementary school in China was investigated, and findings were linked to the presence of Shigella flexneri 2b in untreated well water. A diarrhoea outbreak in China caused by enteropathogenic and enterotoxigenic E. coli was noted among 131 individuals exposed to well water contaminated by river water. Between May and August 2010 in Southern Vietnam, Nguyen and co-authors documented an outbreak of diarrhea that infected seventy-one persons through the consumption of iced tea contaminated with V. cholerae O1, altered El Tor strains. Also, Ali et al. noted a typhoid fever outbreak that affected 1430 individuals, originating from the consumption of fecally contaminated tap water both in military camps and the general population located in Kikwit, Democratic Republic of Congo, in 2011. In a Croatian study, Kovačić et al. reported an outbreak of gastroenteritis, which affected 68 persons in a County Centre of Šibenik, caused by the pollution of groundwater used for drinking with Salmonella enterica subsp. enterica serovar Enteritidis. Fortunately, the isolates of the bacterium were susceptible to a range of antibiotics.

Furthermore, Farooqui et al. documented an outbreak of typhoid fever caused in Nek Muhammad village, Karachi—Pakistan, which involved over 300 hundred people, and resulted in the deaths of three humans in 2004. The findings in this study associated the outbreak to multidrug-resistant Salmonella enterica serovar Typhi ingested through well water that served as the sole source of drinking water in the community, but was unacceptably polluted with dead and decaying bodies of animal, fecal material, and garbage.

Similarly, Ma et al. reported the waterborne outbreak of shigellosis in China caused by Shigella sonnei with resistance to azithromycin, and third class cephalospsorins, due to mph (A) and bla CTX-M-14-harboring IncB/O/K/Z group transmissible plasmid. In addition, Akoachere et al. unravelled the persistence of multidrug-resistant toxigenic Vibrio cholerae O1 in water samples (taps, dug wells, and streams) collected from New Bell—Doula, and associated these bacteria strains with the 2010–2011 cholera epidemic that recorded the highest number of death cases in the Littoral province of Cameroon.

ia are mostly found in animals, animal-derived food products, and agro-food environments. Consequently, most of the gastrointestinal diseases of humans of public health concern including gastroenteritis, salmonellosis, shigellosis, cholera, listeriosis, campylobacteriosis, and yersiniosis are caused by pathogenic bacteria of public health and environmental importance via water and/or food contaminated with feces of animals or patients. These diseases even become more complicated and severe with the presence of the antibiotic-resistant strains of the causative pathogens harbouring antibiotic resistance genes. As a result, Table 2 compiles information on the notorious environmental pathogens causing gastrointestinal and other diseases in humans/animals through the consumption of fecally-contaminated water and/or food, their resistance to antibiotics typical of human, veterinary and agricultural use, and the implicated antibiotic resistance genes occurring in some countries.

Notorious pathogens, antibiotic resistance, and implicated resistance genes from environmental samples in some developing countries.

The data in the tables represent the findings obtained from several studies conducted by different researchers across the world in their quest to determine antibiotic resistance genes in environmental sources, in a bid to strategically fight against the problem of rising antibiotic resistance. These findings were retrieved from published articles found in Science Direct, Pubmed, Medline databases, etc., using Google search engine. The search was done in a systematic way, and the search query term was modified using related terms to maximize accuracy.

Pathogens	Antibiotic Resistance	Resistance Genes	Source	Diseases Caused/Symptoms	Country
		bla $_{CTX-M-9}$, *qnr* S	Drinking water		
		bla $_{CTX}$, *bla* $_{TEM}$	Vegetables, raw eggs, raw chicken, unpasteurized milk, raw meat	Diarrhoea, septicaemia, urinary tract infections, neonatal meningitis, abdominal pain, fever, pneumonia, hemolytic uraemic syndrome, nosocomial bacteraemia	India
Escherichia coli	Multidrug resistance	*bla* $_{CTX-M-15}$, *bla* $_{OXA-1}$, *bla* $_{CMY-2}$, *qnr* S, *qnr* B	Household water supply		Bangladesh
		NA	Bird cloacae		Saudi Arabia
		bla $_{CTX}$, *bla* $_{SHV}$, *bla* $_{TEM}$, *bla* $_{CTX-M}$ + *bla* $_{TEM}$, *bla* $_{CTX-M}$ + *bla* $_{SHV}$, *bla* $_{CTXM}$ + *bla* $_{SHV}$ + *bla* $_{TEM}$, *bla* $_{TEM}$ + *bla* $_{SHV}$	Poultry swab		Zambia
		tet, *dfr* A, *bla* $_{TEM-1}$	Animal feces		Zimbabwe
		Sul I, *Sul* II, *bla* PSE 1, *AmpC*	Zoo lake		Brazil

It can be depicted from Table 2, that antibiotic resistance is a global challenge, and needs to be addressed by the joint efforts from all the countries across the world, as we can never stop international travel and trade. Secondly, antibiotic resistant bacteria and resistance genes do not originate only from humans relating to clinical settings, but they are available and prevalent in the environment, food, and water that we consume. In addition, the table emphasises the presence of multidrug resistance in the respective countries, which is an uglier scenario, as the antibiotic resistance genes can be disseminated via food, water, and other environmental sources to clinical

pathogens, thereby causing health threats to the public (especially the immunocompromised individuals who depend on antibiotics to boost their immune system). Consequently, owing to the high infectious disease burden in developing countries, alongside the poor sanitation and hygienic conditions, data in the tables create more awareness in the population about environmental antibiotic resistance, such that intervention measures be reinforced, to ensure prudent use of antibiotics, in a bid to contain antibiotic resistance. Furthermore, there is a clear need for continuous surveillance to monitor antibiotic resistance.

BIOPESTICIDES

The term biopesticides defines compounds that are used to manage agricultural pests by means of specific biological effects rather than as broader chemical pesticides. It refers to products containing biocontrol agents – i.e., natural organisms or substances derived from natural materials (such as animals, plants, bacteria, or certain minerals), including their genes or metabolites, for controlling pests. According to the FAO definition, biopesticides include those biocontrol agents that are passive agents, in contrast to biocontrol agents that actively seek out the pest, such as parasitoids, predators, and many species of entomopathogenic nematodes. Thus biopesticides cover a wide spectrum of potential products that can be classified as follows: l Microbial pesticides and other entomopathogens: pesticides that contain microorganisms, like bacteria, fungi, or virus, which attack specific pest species, or entomopathogenic nematodes as active ingredients. Although most of these agents attack insect species (called entomopathogens; products referred to as bioinsecticides), there are also microorganisms (i.e., fungi) that control weeds (bioherbicides). l Plant-Incorporated Protectants (PIPs): these include pesticidal substances that are produced in genetically modified plants/organisms (GMO) (i.e., through the genetic material that has been incorporated into the plant). l Biochemical pesticides: pesticides based on naturally occurring substances that control pests by non-toxic mechanisms, in contrast to chemical pesticides that contain synthetic molecules that directly kill the pest. Biochemical pesticides fall into different biologically functional classes, including pheromones and other semiochemicals, plant extracts, and natural insect growth regulators. Biopesticides generally have several advantages compared to conventional pesticides. While chemical pesticides are responsible for extensive pollution of the environment, a serious health hazard due to the presence of their residues in food, development of resistance in targeted insect pest populations, a decrease in biodiversity, and outbreaks of secondary pests that are normally controlled by natural enemies, biopesticides, in contrast, are inherently less toxic to humans and the environment, do not leave harmful residues, and are usually more specific to target pests. Often they affect only the target pest and closely related organisms, substantially reducing the impact on non-target species.

A further advantage of most microbial pesticides is that they replicate in their target hosts and persist in the environment due to horizontal and vertical transmission, which may cause long-term suppression of pest populations even without repeating the application. Since the use of the biopesticides is markedly safer for the environment and users, and more sustainable than the application of chemicals, their use as alternatives to chemical pesticides, especially as components in Integrated Pest Management (IPM) strategies, is of growing interest. Several biopesticides of the different classes have proved to be very effective in controlling potato pests; however, there are certain disadvantages associated with their use that have prevented them from being used on a wider basis in potato production today. The very high specificity of the products might be a disadvantage when a complex of pest species needs to be controlled. Since biopesticides often contain living material, the products have reduced shelf lives. Also, their efficacy is often variable due to the influence of various biotic and abiotic factors. For using biopesticides 168 effectively, users need to have good knowledge about managing the particular pests or pest complexes. Due to limited commercial use (niche products) biopesticides often are developed by research institutions rather than by the traditional pesticide industry. While effective active ingredients have been discovered, products might lack appropriate formulation for efficient field use. A broader set of perspectives in the design and launch of a biopesticide would be helpful. Farmers consider biopesticides often as an alternative to a chemical pesticide, in which the active ingredient is thought to be synthetic, having a similar mode of action to the chemical pesticide. But the truth is that biopesticides differ in their modes of action from conventional chemical pesticide considerably; their modes of action are almost always specific. Therefore, using biopesticides efficiently requires specific user knowledge on the agent and the target pest for optimizing application time, field rates, and application intervals. Biopesticides should not be considered as a one-for-one replacement of chemical pesticides. As pesticides in general, biopesticides need to be approved and registered as such in most countries before they can be used, sold, or supplied. Since biopesticides pose fewer risks than conventional pesticides, authorities generally require fewer data for their registration. For example, the Environmental Protection Agency (EPA) in the USA often registers new biopesticides in less than a year, compared with an average of more than 3 years for conventional pesticides. However, in some cases it is difficult to determine whether a product meets the criteria for classification as a biopesticide, and the decision by local agencies might vary depending on the regulations in each country. There might be specific requirements pertinent to the different categories of biopesticides. In this chapter, the major biopesticides of potato pests and their potential for integrated pest management are reviewed according to their categories. Because knowledge about the mode of action of each type of active ingredient is crucial, each subsection includes a brief description of the biocontrol agent.

Factors	Benefits of biopesticides
Cost effectiveness	Costlier but reduced number of applications
Persistence and residual effect	Low, mostly biodegradable and self perpetuating
Knockdown effect	Delayed
Handling and Bulkiness	Bulky: Carrier based Easy:Liquid formulation
Pest resurgence	Less
Resistence	Less prone
Effect on beneficial flora	Less harmful on beneficial pests
Target specificity	Mostly host specific
Waiting time	Almost nil
Nature of control	Preventive
Shelf life	Less

Biopesticides fall into three major categories:

(1) Microbial pesticides contain a microorganism (bacterium, fungus, virus, protozoan or alga) as the active ingredient. Microbial pesticides can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest[s]. For example, there are 169 fungi that control certain weeds, and other fungi that kill specific insects. The most widely known microbial pesticides are varieties of the bacterium Bacillus thuringiensis, or Bt, which can control certain insects in cabbage, potatoes, and other crops. Bt produces a protein that is harmful to specific insect pests. Certain other microbial pesticides act by out-competing pest organisms. Microbial pesticides need to be continuously monitored to ensure they do not become capable of harming non-target organisms, including humans.

(2) Plant-pesticides are pesticidal substances that plants produce from genetic material that has been added to the plant. For example, scientists can take the gene for the Bt pesticidal protein, and introduce the gene into the plants own genetic material. Then the plant, instead of the Bt bacterium manufactures the substance that destroys the pest. Both the protein and its genetic material are regulated by EPA; the plant itself is not regulated.

(3) Biochemical pesticides are naturally occurring substances that control pests by non-toxic mechanisms. Conventional pesticides, by contrast, are synthetic materials that usually kill or inactivate the pest. Biochemical pesticides include substances that interfere with growth or mating, such as plant growth regulators, or substances that repel or attract pests, such as pheromones. Because it is sometimes difficult to determine whether a natural pesticide controls the pest by a non-toxic mode of action, EPA has established a committee to determine whether a pesticide meets the criteria for a biochemical pesticide.

The growth of total world production of biopesticides is rising and therefore demand and use is also increasing. In India, biopesticide consumption has shown its increased use over the time. In 2005-06, consumption of biopesticides in India stands at 1920 MT.

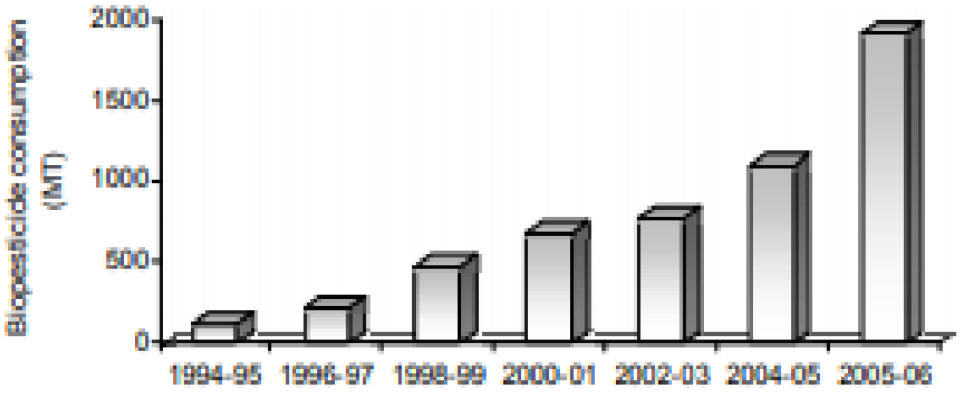


Figure 1. Consumption of biopesticides (MT)

India has a vast potential for biopesticides. However, its adoption by farmers in India needs education for maximizing gains. Biopesticides represent only 2.89% (as on 2005) of the overall pesticide market in India and is expected to exhibit an annual growth rate of about 2.3% in the coming years (Thakore, 2006). In India, so far only 12 types of biopesti cides have been 170 registered under the Insecticide Act, 1968.

Neem

Derived from the neem tree (Azadirachta indica), this contains several chemicals, including 'azadirachtin', which affects the reproductive and digestive process of a number of important pests. Recent research carried out in India and abroad has led to the development of effective formulations of neem, which are being commercially produced. As neem is non-toxic to birds and mammals and is non-carcinogenic, its demand is likely to increase. However, the present demand is very small. Although more than 100 firms are registered to produce neem-based pesticides in India, only a handful are actually producing it. Furthermore, very little of the production is sold locally, most being for export markets.

Bacillus thuringiensis (Bt)

Bacillus thuringiensis is the most commonly used biopesticide globally. It is primarily a pathogen of lepidopterous pests like American bollworm in cotton and stem borers in rice. When ingested by pest larvae, Bt releases toxins which damage the mid gut of the pest, eventually killing it. Main sources for the production of BT preparations are the strains of the subspecies kurstaki, galeriae and dendrolimus.

Mode of Action

Unlike many synthetic insecticides, the insecticidal proteins produced by *Bt* strains are very specific for certain insect species. For example, some only affect caterpillars while others affect beetles, but none of them affect mammals. The many proteins produced are named and categorized based on their primary amino acid sequence and the type of insects they affect. Because of the narrow spectrum of activity against target insects, they are considered environmentally safe for birds, mammals, natural insect enemies that help suppress pest populations, and aquatic animals. For these reasons, the applications of these bio-insecticidal proteins are widespread and the topical formulations are used in organic farms. However, persistent use of topical solutions in an agricultural setting has led to cases of resistance in several species. Additionally, there are a few cases of insects having evolved resistance to transgenic plants expressing *Bt* proteins (Tabashnik et al. 2013).

Crystalline Toxin Mode of Action

Upon ingestion, the *Bt* protein is activated by alkaline (pH>7)-dependent proteins, which change the *Bt* protein into an active form within the insect's digestive tract. This activation allows the protein to bind to receptors located on the intestinal lining. Once the protein binds, it forms holes in the gut that lead to cell death, septicemia, and insect mortality. The dead insect then supplies a nutrient- rich environment for the bacteria to propagate (Bravo et al. 2011).

Changes in protein activation, diffusion, or receptor binding have all been associated with resistance in various insect pests. The first species to exhibit resistance to *Bt* toxins in an agricultural setting was the diamondback moth, *Plutella xylostella* (Syed, 1990). Populations in Malaysia and the Philippines that showed resistance were immune to the *Btk* derived formulation Dipel®, which expresses Cry1Aa, Cry1Ab, Cry1Ac, Cry2A and Cry2Ab (Iqbal et al., 1996). With increased use of *Bt* sprays, resistance to *Bta* and *Btk* formulations arose in Hawaii, Pennsylvania, Florida, and the Philippines (Shelton et al., 1993). The mechanisms of resistance in *P. xylostella* have been extensively investigated and were shown to have similar qualities between geographically isolated populations (Tabashnik et al. 1997). To categorize this phenomenon, Tabashnik devised a system to objectively differentiate a type of *Bt* resistance known as Mode 1 from other heritable forms of resistance.

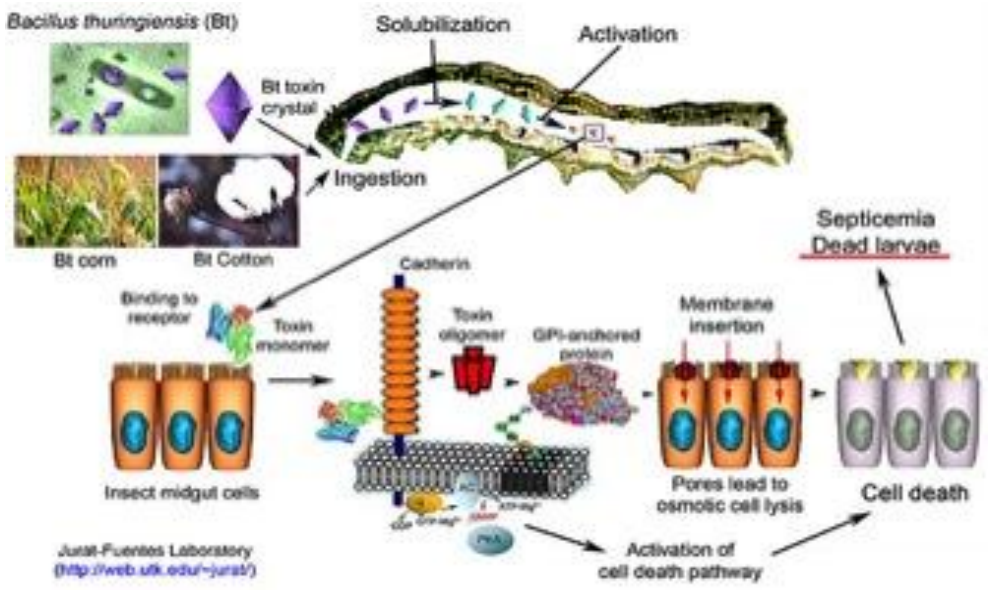


Figure 3. Insecticidal protein mode of action. [http://web.utk.edu/~jurat/Btresearchtable.html]

Mode 1 resistance is characterized by >500-fold resistance to at least one Cry1A protein via reduced binding at the midgut membrane, with little cross-resistance to other *Bt* proteins (Tabashnik et al. 1998). Diamondback moth strains from Hawaii, Pennsylvania, Florida, the Philippines, and South Carolina have developed resistance to *Bt* sprays and all exhibit characteristics of Mode 1 resistance (Tabashnik et al 1998). Non-Mode 1 resistance to *Bt* crops has arisen in three insect pest species of corn: Western corn rootworm, *D. virgifera virgifera*, fall armyworm, *Spodoptera frugiperda,* and maize stalk borer, *Busseola fusca;* and one to transgenic cotton: pink bollworm, *Pectinophora gossypiella* (Tabashnik et al 2013). Only resistance in one pest, *S. frugiperda,* in Puerto Rico has caused the crop to be taken off the market (Moar et al. 2008). However, strong resistance to Cry1F corn among populations of this species has recently caused crop loss in Brazil (Farias et al., 2014).

Baculoviruses

These are target specific viruses which can infect and destroy a number of important plant pests. They are particularly effective against the lepidopterous pests of cotton, rice and vegetables. Their large-scale production poses certain difficulties, so their use has been limited to small areas. They are not available commercially in India, but are being produced on a small scale by various IPM centres and state agricultural departments.

Baculoviruses are pathogens that attack insects and other arthropods. Like some human viruses, they are usually extremely small (less than a thousandth of a millimeter across), and are composed primarily of double-stranded DNA that codes for genes needed for virus establishment and reproduction. Because this genetic material is easily destroyed by exposure to sunlight or by conditions in the host's gut, an infective baculovirus particle (*virion*) is protected by protein coat

called a *polyhedron* (plural *polyhedra*: see Figs. *A, B, and C*). Most insect baculoviruses must be eaten by the host to produce an infection, which is typically fatal to the insect.

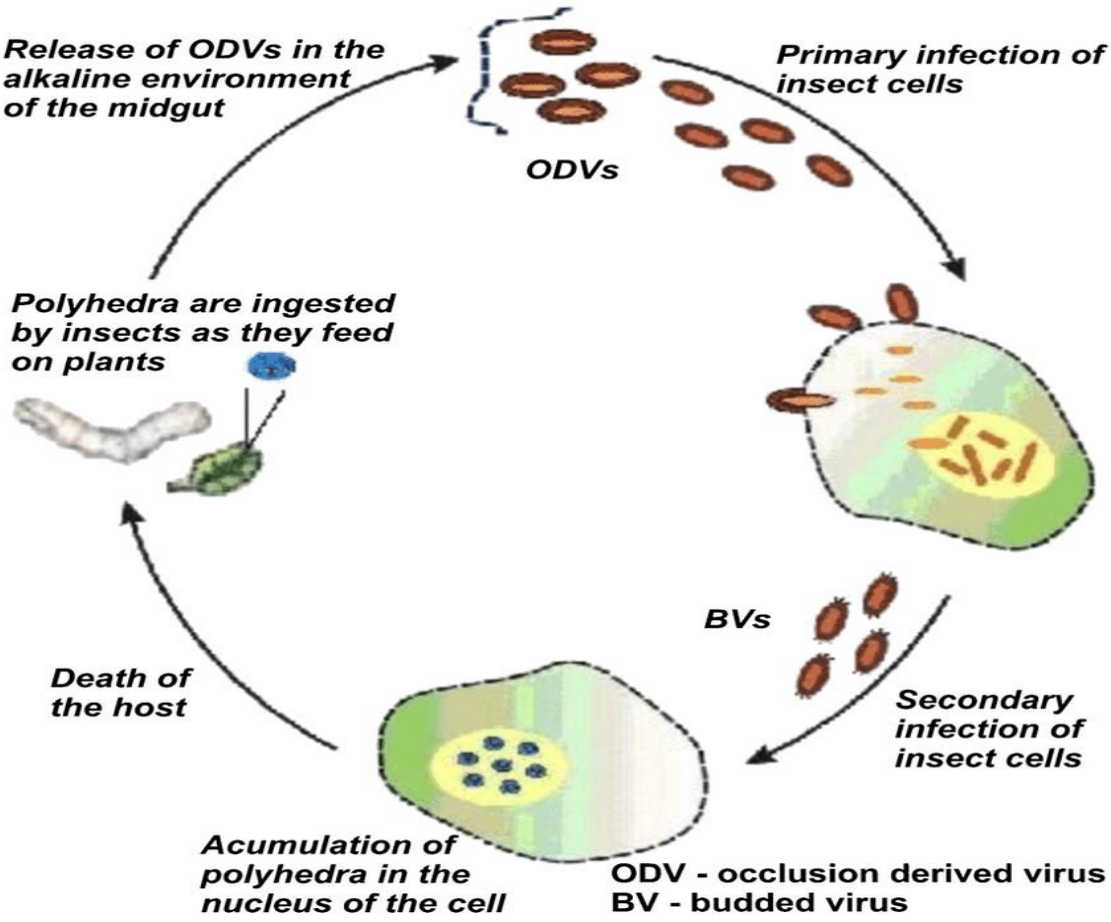
The majority of baculoviruses used as biological control agents are in the genus *Nucleopolyhedrovirus*, so "baculovirus" or "virus" will hereafter refer to nucleopolyhedroviruses. These viruses are excellent candidates for species-specific, narrow spectrum insecticidal applications. They have been shown to have no negative impacts on plants, mammals, birds, fish, or even on non-target insects. This is especially desirable when beneficial insects are being conserved to aid in an overall IPM program, or when an ecologically sensitive area is being treated. The USDA Forest Service currently uses the gypsy moth nuclear polyhedrosis virus (*LdNPV*) to aerially spray thousands of acres of forest each year. This product, registered as **Gypchek**, is effective against gypsy moths but leaves all other animals unharmed (Reardon et al. 1996).

On the other hand, the high specificity of baculoviruses is also cited as a weakness for agricultural uses, since growers may want one product to use against a variety of pests. Currently, researchers are attempting to use genetic engineering techniques to expand virus host ranges to the desired pest species. Releases of such genetically-engineered baculoviruses have been made by researchers in the U.K. and the United States and show promise, although the cost of commercial production of these agents must be reduced if they are to be competitive. Companies like Dupont, biosys, American Cyanamid, and Agrivirion (to name a few) have continued to explore the expansion and development of agricultural-use viral insecticides. Recently, biosys has released two baculovirus-based products, Spod-X for beet armyworm and Gemstar LC for tobacco budworm and cotton bollworm.

Life Cycle

Viruses are unable to reproduce without a host - they are *obligate parasites*. Baculoviruses are no exception. The cells of the host's body are taken over by the genetic message carried within each virion (Fig. C), and forced to produce more virus particles until the cell, and ultimately the insect, dies. Most baculoviruses cause the host insect to die in a way that will maximize the chance that other insects will come in contact with the virus and become infected in turn (above and Fig. D).

As seen in the animation on the right, infection by baculovirus begins when an insect eats virus particles on a plant - perhaps from a sprayed treatment. The infected insect dies and "melts" or falls apart on foliage, releasing more virus. This additional infective material can infect more insects, continuing the cycle.



Natural life cycle of baculovirus AcMNPV. Polyhedra are taken orally by the larvae with plant material and are dissolved in the alkaline environment of the midgut. ODVs are liberated and infect epithelial mi dgut cells. Virus replicates and budded viruses (BVs) are produced.

Trichoderma

Trichoderma is a fungicide effective against soil born diseases such as root rot. It is particularly relevant for dryland crops such as groundnut, black gram, green gram and chickpea, which are susceptible to these diseases. Preparation of Trichoderma biopesticide is cheap and requires only basic knowledge of microbiology.

Trichogramma

Trichogramma are minute wasps which are exclusively eggparasites. They lay eggs in the eggs of various lepidopteran pests. After hatching, the Trichogramma larvae feed on and destroy the host egg. Trichogramma is particularly effective against lepidopteran pests like the sugarcane internode borer, pink bollworm and sooted bollworms in cotton and stem borers in rice. They are also used against vegetable and fruit pests.

Trichogramma is the most popular biocontrol agent in India, mainly because it kills the pest in the egg stage, ensuring that the parasite is destroyed before any damage is done to the crop. Trichogramma eggs have to be used within a short period (before the eggs hatch). This limits their production and marketing on a large scale, and is also the reason why Trichogramma is not sold through dealers and shopkeepers.

Some success stories about successful utilization of biopesticides and bio-control agents in Indian agriculture include:

1. Control of diamondback moths by Bacillus thuringiensis,
2. Control of mango hoppers and mealy bugs and coffee pod borer by Beauveria,
3. Control of Helicoverpa on cotton, pigeon-pea, and tomato by Bacillus thuringiensis,
4. Control of white fly on cotton by neem products,
5. Control of Helicoverpa on gram by N.P.V.,
6. Control of sugarcane borers by Trichogramma and
7. Control of rots and wilts in various crops by Trichoderma-based products.

Opportunities

The area under organic cultivation (crops) in India is estimated to be around 1,00,000 hectare. Besides, there are lakhs of hectare of forest area being certified as organic. Further, some states like Uttaranchal and Sikkim have declared their states as organic. Moreover, the area under organic crop cultivation may rise because of the growing demand of organic food, a result of increasing health consciousness among the people. This indicates that there is huge scope for growth of the biopesticide sector in India. At the same time increasing population can be fed by organic farming dependence is a big question and unless organic farming yield can be brought equal to that of conventional farming involving the use of agrochemicals etc, the organic farming may not be feasible at the moment. Analysts believe that there would be a greater development in the biopesticides sector. Due to its rich biodiversity India offers plenty of scope in terms of sources for natural biological control organisms as well as natural plant based pesticides. The rich traditional knowledge base available with the highly diverse indigenous communities in India may provide valuable clues for developing newer and effective biopesticide. The stress on organic farming and on residue free commodities would certainly warrant increased adoption of biopesticides by the farmers. Increased adoption further depends on1.Concrete evidences of efficacy of biopesticides in controlling crop damage and the resultant increase in crop yield, 2. Availability of high quality products at affordable prices, 3. Strengthning of supply chain management in order to increase the usage of biopesticides. In this regard, an efficient delivery system from the place of production

(factory) to place of utilization (farm) of biopesticides is quite essential. The National Farmer Policy 2007 has strongly recommended the promotion of biopesticides for increasing agricultural production, sustaining the health of farmers and environment. It also includes the clause that biopesticides would be treated at par with chemical pesticides in terms of support and promotion. Further research and 172 development of biological pest control methods must be given priority and people in general and agriculturists in particular must be educated about the handling and use of such control measures. All this will lead to a general understanding about the benefits of biopesticides as green alternative. However, the need in the present day context is on IPM, INM, ICM and GAP and by practicing these the quality of life and health will be assured.

BIOFERTILIZER

A biofertilizer (also bio-fertilizer) is a substance which contains living microorganisms which, when applied to seeds, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. Biofertilizers add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus, and stimulating plant growth through the synthesis of growthpromoting substances. Biofertilizers can be expected to reduce the use of synthetic fertilizers and pesticides. The microorganisms in biofertilizers restore the soil's natural nutrient cycle and build soil organic matter. Through the use of biofertilizers, healthy plants can be grown, while enhancing the sustainability and the health of the soil. Since they play several roles, a preferred scientific term for such beneficial bacteria is "plant-growth promoting rhizobacteria" (PGPR). Therefore, they are extremely advantageous in enriching soil fertility and fulfilling plant nutrient requirements by supplying the organic nutrients through microorganism and their byproducts. Hence, biofertilizers do not contain any chemicals which are harmful to the living soil.

Biofertilizers provide "eco-friendly" organic agro-input. Biofertilizers such as Rhizobium, Azotobacter, Azospirilium and blue green algae (BGA) have been in use a long time. Rhizobium inoculant is used for leguminous crops. Azotobacter can be used with crops like wheat, maize, mustard, cotton, potato and other vegetable crops. Azospirillum inoculations are recommended mainly for sorghum, millets, maize, sugarcane and wheat. Blue green algae belonging to a general cyanobacteria genus, Nostoc or Anabaena or Tolypothrix or Aulosira, fix atmospheric nitrogen and are used as inoculations for paddy crop grown both under upland and low-land conditions. Anabaena in association with water fern Azolla contributes nitrogen up to 60 kg/ha/season and also enriches soils with organic matter.

Other types of bacteria, so-called phosphate-solubilizing bacteria, such as Pantoea agglomerans

strain P5 or Pseudomonas putida strain P13, are able to solubilize the insoluble phosphate from organic and inorganic phosphate sources. In fact, due to immobilization of phosphate by mineral ions such as Fe, Al and Ca or organic acids, the rate of available phosphate (Pi) in soil is well below plant needs. In addition, chemical Pi fertilizers are also immobilized in the soil, immediately, so that less than 20 percent of added fertilizer is absorbed by plants. Therefore, reduction in Pi resources, on one hand, and environmental pollutions resulting from both production and applications of chemical Pi fertilizer, on the other hand, have already demanded the use of phosphate-solubilizing bacteria or phosphate biofertilizers.

Types:

Nitrogen Biofertilizers:- This type of biofertilizers helps the agriculturists to determine the nitrogen level in the soil. Nitrogen is a necessary component which is used for the growth of the plant. Plants need a limited amount of nitrogen for their growth. The type of the crops also determines the level of nitrogen. Some crops need more nitrogen for their growth while some crops need fewer amounts. The type of the soil also determines that which type of biofertilizers is needed for this crop. Fr example, Azotobacteria is used for the non legume crops; Rhizobium is needed for the legume crops. Similarly blue green algae are needed to grow rice while Acetobacter is used to grow sugarcane.

Phosphorus biofertilizers:- Phosphorus biofertilizers are used to determine the phosphorus level in the soil. The need of phosphorus for the plant growth is also limited. Phosphorus biofertilizers make the soil get the required amount of phosphorus. It is not necessary that a particular phosphorus biofertilizers is used for a particular type of crop. They can be used for any types of the crops for example; Acetobacter, Rhizobium and other biofertilizers can use phosphotika for any crop type.

Compost Biofertilizers:- Compost biofertilizers are those which make use of the animal dung to enrich the soil with useful microorganisms and nutrients. To convert the animals waste into a biofertilizers, the microorganisms like abcteria undergo biological processes and help in breaking down the waste. Cellulytic fungal culture and Azetobacter cultures can be used for the compost biofertilizers.

Advantages of biofertilizers:-

1) They help to get high yield of crops by making the soil rich with nutrients and useful microorganisms necessary for the growth of the plants.

2) Biofertilizers have replaced the chemical fertilizers as chemical fertilizers are not beneficial for the plants. They decrease the growth of the plants and make the environment polluted by releasing

harmful chemicals.

3) Plant growth can be increased if biofertilizers are used, because they contain natural components which do not harm the plants but do the vice versa.

4) If the soil will be free of chemicals, it will retain its fertility which will be beneficial for the plants as well as the environment, because plants will be protected from getting any diseases and environment will be free of pollutants.

5) Biofertilizers destroy those harmful components from the soil which cause diseases in the plants. Plants can also be protected against drought and other strict conditions by using biofertilizers.

6) Biofertilizers are not costly and even poor farmers can make use of them.

7) They are environment friendly and protect the environment against pollutants.

Applications of biofertilizers to crop:-

Seedling root dip:- This method is applied to the rice crop. A bed of water is spread on the land where the crop has 174 to grow. The seedlings of rice are planted in the water and are kept there for eight to ten hours.

Seed treatment:- In this method, the nitrogen and phosphorus fertilizers are mixed together in the water. Then seeds are dipped in this mixture. After the applications of this paste to the seeds, seeds are dried. After they dry out, they have to be sown as soon as possible before they get damaged by harmful microorganisms.

Soil treatment:- All the biofertilizers along with the compost fertilizers are mixed together. They are kept for one night. Then the next day this mixture is spread on the soil where seeds have to be sown.

Liquid bio-fertilizer application methodology

• Seed treatment: Seed treatment is the most common method adopted for all types of inoculants.

• The seed treatment is effective and economic. For small quantities of seeds (up to 5 kg), the coating can be done in a plastic bag.

• For this purpose, a plastic bag sized 21” x 10” or larger can be used. The bag should be filled with 2 kg or more of seeds.

• The bag should be closed in such a way so as to trap the air as much as possible.

• The bag should be squeezed for 2 minutes or more until all the seeds are uniformly wetted.

• Then the bag is opened, inflated again and shaken gently. The shaking can stop after each seed gets a uniform layer of culture coating.

• The bag is opened and the seeds are dried in the shade for 20–30 minutes. ...

Liquid bio-fertilizer application methodology Root dipping:

This method is used for application of Azospirillum/PSM on paddy transplanting/ vegetable crops. • The required quantity of Azospirillum /PSM has to be mixed with 5–10 liters of water at one corner of the field and the roots of seedlings have to be dipped for a minimum of half-an-hour before transplantation. Soil application: Use 200ml of PSM per acre. • Mix PSM with 400 to 600 kgs of cow dung FYM (farmyard manure) along with ½ bag of rock phosphate if available. • The mixture of PSM, cow dung and rock phosphate has to be kept under any tree or in the shade overnight and 50% moisture should be maintained. • The mixture is used for soil application in rows or during leveling of soil.

PRECAUTIONS BEFORE BIOFERTILIZER APPLICATION

• Biofertilizer packets need to be stored in a cool and dry place away from direct sunlight and heat. • Right combinations of biofertilizers have to be used. • As Rhizobium is crop specific, one should use it for the specified crop only. • Other chemicals should not be mixed with the biofertilizers. • When purchasing, one should ensure that each packet is provided with all necessary information like name of the product, name of the crop for which it is intended, name and address of the manufacturer, date of manufacture, date of expiry, batch number and instructions for use. • The packet has to be used before its expiry, only for the specified crop and by the recommended method of application. • Biofertilizers are live products and require care in their storage. • It is important to use biofertilizers along with chemical fertilizers and organic manures. Biofertilizers are not a replacement of fertilizers but can supplement plant nutrient requirements. Role of Biofertilizers in Agriculture • Biofertilizers supplement synthetic fertilizers and fulfil the nutrient requirement of crops. • Bio-fertilizers add 20-180 kg N/ha in soil and enhance crop production and nutrient use efficiency in a particular optimum environment. • They efficiently reduce use of synthetic fertilizers and create chemical free yield. • Application of bio-fertilizers results in improved nutrient and water uptake, soil quality, rhizosphere development etc. • These bio-fertilizers promote growth of plant through release of growth simulating substances. • These bio-fertilizers includes variety of micro-organism which successfully reduces harmful pathogens resulting various diseases thus control many diseases. • Bio-fertilizers improve soil fertility, physical properties of soil, tilth and crop- productivity. **Factors Affecting Bio-Fertilizer Response**

• Efficiency of any inoculant and micro-organisms to be determined by host plant and genotype. • Quality of inoculant largely influences its results in term of nitrogen fixation and solubilisation of particular nutrients. • Package of practices and management of crop alter results of bio- fertilizers. • Soil physical and chemical properties highly influence impact of different inoculants and micro-organisms.

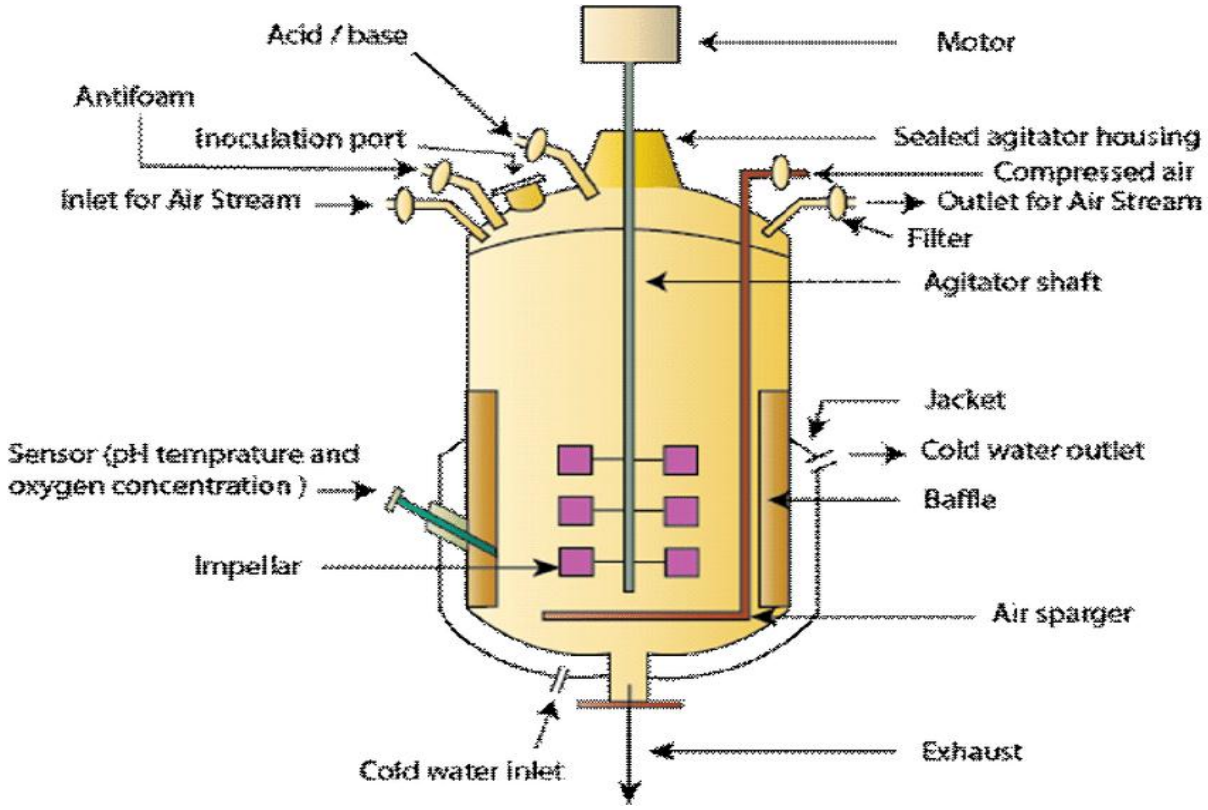
• Climatic conditions like temperature, relative humidity, rainfall and photoperiod affect response of biofertilizers significantly. Constraints in Bio-Fertilizer Application • There is lack of good quality of strain which efficiently provide required nutrients in soil. • Non- existence of storage facility makes it difficult to adopt bio-fertilizers. • Field conditions like extremely high or low pH, temperature, nutrients deficiency not only influence the response of inoculants but also limits heir benefits.

7. Industrial Microbiology

Fermenters- stirred tank, bubble column, air lift, packed bed

Stirred Tank Fermenter

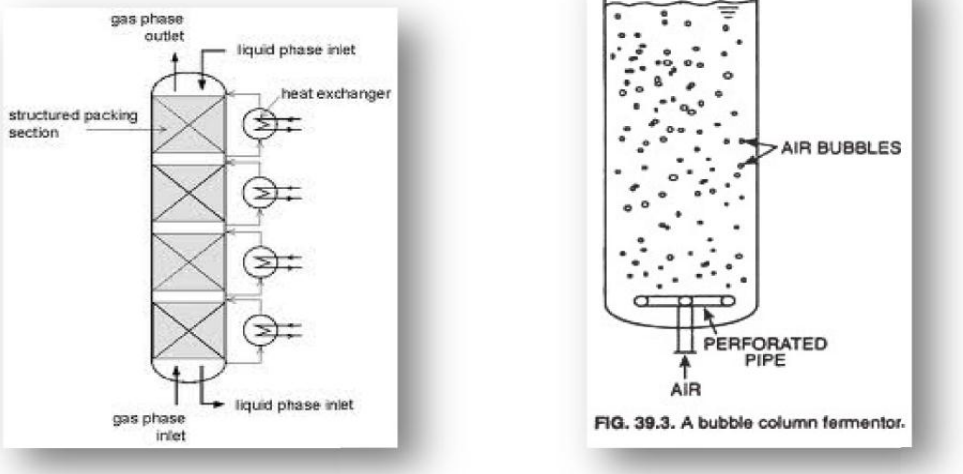
Microbial fermentations received prominence during 1940's namely for the production of life saving antibiotics. Stirred tank reactor is the choice for many (more than 70%) though it is not the best. Stirred tank reactor's have the following functions: homogenization, suspension of solids, dispersion of gas-liquid mixtures, aeration of liquid and heat exchange. The Stirred tank reactor is provided with a baffle and a rotating stirrer is attached either at the top or at the bottom of the bioreactor. The typical decision variables are: type, size, location and the number of impellers; sparger size and location. These determine the hydrodynamic pattern in the reactor, which in turn influence mixing times, mass and heat transfer coefficients, shear rates etc. The conventional fermentation is carried out in a batch mode. Since stirred tank reactors are commonly used for batch processes with slight modifications, these reactors are simple in design and easier to operate. Many of the industrial bioprocesses even today are being carried out in batch reactors though significant developments have taken place in the recent years in reactor design, the industry, still prefers stirred tanks because in case of contamination or any other substandard product formation the loss is minimal. The batch stirred tanks generally suffer due to their low volumetric productivity. The downtimes are quite large and unsteady state fermentation imposes stress to the microbial cultures due to nutritional limitations. The fed batch mode adopted in the recent years eliminates this limitation. The Stirred tank reactor's offer excellent mixing and reasonably good mass transfer rates. The cost of operation is lower and the reactors can be used with a variety of microbial species. Since stirred tank reactor is commonly used in chemical industry the mixing concepts are well developed. Stirred tank reactor with immobilized cells is not favored generally due to attrition problems; however by separating the zone of mixing from the zone of cell culturing one can successfully operate the system.



Continuous stirred-

tank reactors (Fermenter: Equipment design) (Source: nptel@iitm.ac.in) CSTRs consist of a tank, usually of constant volume, and a stirring system to mix reactants together. Also, feed and exit pipes are present to intr oduce reactants and remove products.

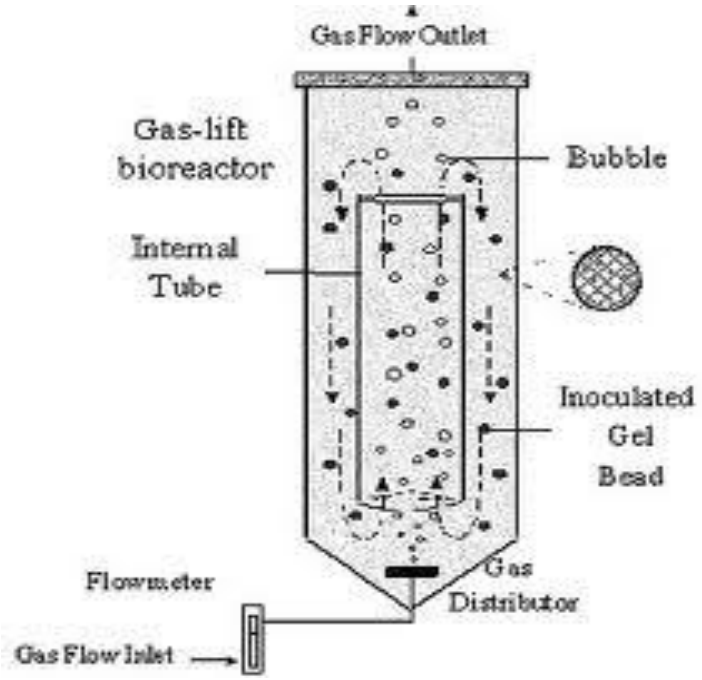
Bubble Column Bioreactor



Diagrammatic views of a Bubble Column Fermentor

In the bubble column bioreactor, the air or gas is introduced at the base of the column through perforated pipes or plates, or metal micro The bubble column bioreactors may be fitted with perforated plates to improve performance. The vessel used for bubble column bioreactors is usually cylindrical with an aspect ratio of 4-6 (i.e., height to diameter ratio). mixing and other performance factors are influenced mainly by the gas flow rate and the rheological properties of the fluid. Internal devices such as horizontal perforated plates, vertical baffles and corrugated sheet packing's may be placed in the vessel to improve mass transfer and modify the basic design. The column diameter does not affect its behavior as long as diameter exceeds 0.1 m. One exception is the axial mixing performance. For a given gas flow rate, the mixing improves with increasing vessel diameter. Mass and heat transfer and the prevailing shear rate increase increased.

Air-Lift Fermenter



Airlift fermenter (ALF) is generally classified as pneumatic reactors without any mechanical stirring arrangements for mixing. The turbulence caused by the fluid flow ensures adequate mixing of the liquid. The draft tube is provided in the central section of the reactor. The introduction of the fluid (air/liquid) causes upward motion and results in circulatory flow in the entire reactor. The air/liquid velocities will be low and hence the energy consumption is also low. ALFs can be used for both free and immobilized cells. There are very few reports on ALFs for metabolite production. The advantages of Airlift reactors are the elimination of attrition effects generally encountered in mechanical agitated reactors. It is ideally suited for aerobic cultures since oxygen mass transfer

coefficient are quite high in comparison to stirred tank reactors. This is ideal for SCP production from methanol as carbon substrate. This is used mainly to avoid excess heat produced during mechanical agitation.

Packed Bed Bioreactor

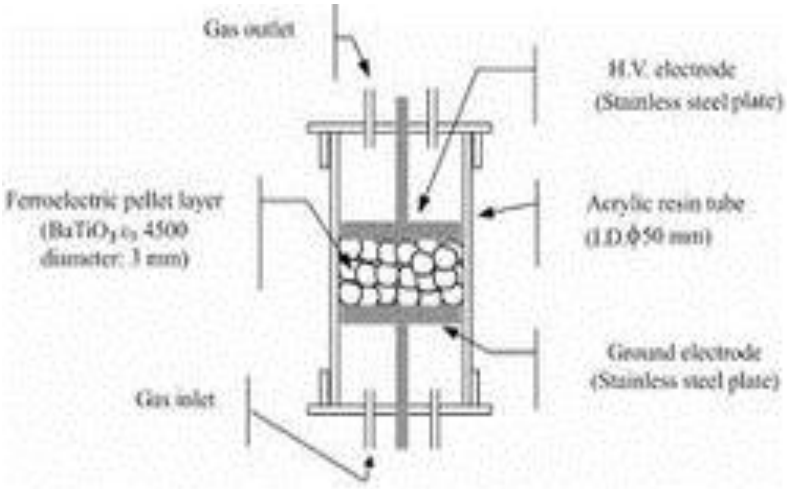


Figure 1: Schematic diagram of the packed-bed reactor.

Packed bed or fixed bed bioreactors are commonly used with attached biofilms especiallyin wastewater engineering. The use of packed bed reactors gained importance after the potential of whole cell immobilization technique has been demonstrated. The immobilized biocatalyst is packed in the column and fed with nutrients either from top or from bottom. One of the disadvantages of packed beds is the changed flow characteristic due to alterations in the bed porosity during operation. While working with soft gels like alginates, carragenan etc the bed compaction which generally occurs during fermentation results in high pressure drop across the bed. In many cases the bed compaction was so severe that the gel integrity was severely hampered. In addition channeling may occur due to turbulence in the bed. Though packed beds belong to the class of plug flow reactors in which backmixing is absent in many of the packed beds slight amount of backmixing occurs which changes the characteristics of fermentation. Packed beds arc generally used where substrate inhibition governs the rate of reaction. The packed bed reactors are widely used with immobilized cells. Several modifications such as tapered beds to reduce the pressure drop across the length of the reactor, inclined bed, horizontal bed, rotary horizontal reactors have been tried with limited success.

• Industrial production of ethyl alcohol, acetic acid, penicillin, vitamin B_{12} and amylase.

Ethanol production:

Ethanol (Ethyl alcohol) CH3CH2OH may be produced by either synthetic chemical method or by fermentation. Ethanol (also called bioethanol) is produced by fermentation from a glucose or sucrose rich medium, the production of alcohol occurs best in the absence of oxygen. The most common ethanologenic microorganisms are yeasts, which include Saccharomyces cerevisiae, Schizosaccharomyces spp., Candida spp., Kluyveromyces lactis, Pichia spp., etc. Bacteria such as Zymomonas mobilis, Clostridium sporogenes, and Leuconostoc mesenteroides are also involved in alcoholic fermentation. The yeast involved in these alcoholic fermentation is mostly strains of Saccharomyces cerevisiae, which cannot directly ferment starch.

Uses of Ethanol

(1) Use as a chemical feed stock: In the chemical industry, ethanol is an intermediate in many chemical processes.

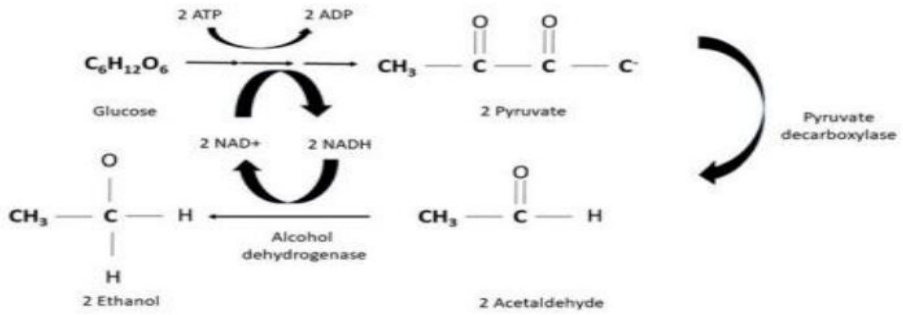
(2) Solvent use: Ethanol is widely used in industry as a solvent for dyes, oils, waxes, cosmetics etc.

(3) General utility: Alcohol is used as a disinfectant in hospitals, for cleaning and lighting in the home, and in the laboratory second only to water as a solvent.

(4) Fuel: Ethanol is mixed with petrol or gasoline up to 10% and known as gasohol.

Biochemistry of ethanol production

The process starts with the breakdown of sugars into pyruvic acid via the Embden Meyerhof-Parnas (EMP) pathway, and then converted into acetaldehyde under anaerobic conditions through the enzyme pyruvate decarboxylase. Acetaldehyde further releases two molecules of carbon dioxide and forms ethanol by alcohol dehydrogenase.



Overall reaction is:

$$\underset{C_6H_{12}O_6}{\text{glucose}} \rightarrow \underset{2\ C_2H_6O}{2\ \text{ethanol}} + 2\ CO_2$$

Yeast grows rapidly in a glucose- or sucrose-rich medium, with a doubling time of about 60 minutes. High growth rates result in O_2 depletion and the neutral reaction products allow growth to be continued anaerobic mode.

Key points

- Ethanol is the end point of the only metabolic pathway that yeast can use for ATP production.
- ATP is produced at a low rate (2 ATP) and 2 moles of ethanol produced per mole of glucose, so that substrate is rapidly converted to product to provide the energy needs of cell growth.
- Ethanol can escape into the medium rather than accumulating in the cell. This makes recovery of the product easier. Pure ethanol has to be recovered by distillation, which could represent the most expensive component of the production process.
-Yield considerations: 1 ton of glucose can be converted into less than 0.5 ton of ethanol.

Ethanol Production process:

- Preparation of the medium

Mashing (Saccharification), this means the grain starch is hydrolyzed to sugars with microbial enzymes or with the enzymes of barley malt. In all the others no hydrolysis is necessary as sugars are present in the fermenting substrate as in grape sugar and cane sugar.

First, starch should be exposed to contact with water. Grinding makes small pieces, which can increase its surface area. Then, the increase in its surface area can enhance the contact between starch and water. At the grinding step, the outer shell of grain is almost completely removed, this step increases the surface area of each particle. These results make water penetrate through grain easily. However, there is still some undesirable area, which is hard to absorb water. To achieve effective enzymatic action, this micro-crystalline area should be removed.

This is typically accomplished with two hot water processes. First, grain is treated with hot water, typically 85°C for between 20 to 60 minutes. Then, super-heated water, typically 110°C, is introduced with high pressure. With the first mixing with hot water, the starch absorbs water. Then, the structure of the micro-crystalline area becomes weak. Then, after introduction of super-heated water with high pressure, this area is completely broken. Without this water treatment, this area cannot be broken, which means the efficiency of the enzymatic action is lost. In an ethanol industry, two enzymes are usually employed, endoenzyme alphaamylase and exoenzyme glucoamylase. Alpha-amylase attacks the alpha-1,4 linkages of starch. Then, starch is converted into dextrin.

After the first hydrolysis with alpha-amylase, glucoamylase works. Glucoamylase removes one glucose from dextrin. Thus, glucoamylase cuts linkage of dextrin from its end.

- Propagation of yeast inoculum

In general the inoculum is made of selected alcohol-tolerant yeast strains usually *Saccharomyces cerevisiae* grown aerobically with agitation and in a molasses base. Yeast inoculum with up to 5% (v/v). Progressively larger volumes of culture may be developed before the desired volume is attained. –

Fermentation:

Yeast is a facultative anaerobe. In the aerobic environment, it converts sugars into carbon dioxide and water, while in the anaerobic environment, it converts sugars into carbon dioxide and ethanol. Thus, for an ethanol industry, it is important to exclude significant oxygen from its system.

Alcohol-resistant yeasts, strains of *Saccharomyces cerevisiae* are used, and nutrients such as nitrogen and phosphate lacking in the broth are added. When the nitrogen content of the medium is insufficient nitrogen is added usually in the form of an ammonium salt. In all alcohol fermentations the heat released must be reduced by cooling and temperatures are generally not permitted to exceed 35- 37°C. The pH is usually in the range 4.5-5.0, when the buffering capacity of the medium is high, higher pH values tend to lead to higher glycerol formation. When the buffering capacity is lower, the pH is falls to about 3.5., the contaminations can have serious effects on the process (sugars are used up leading to reduced yields). –

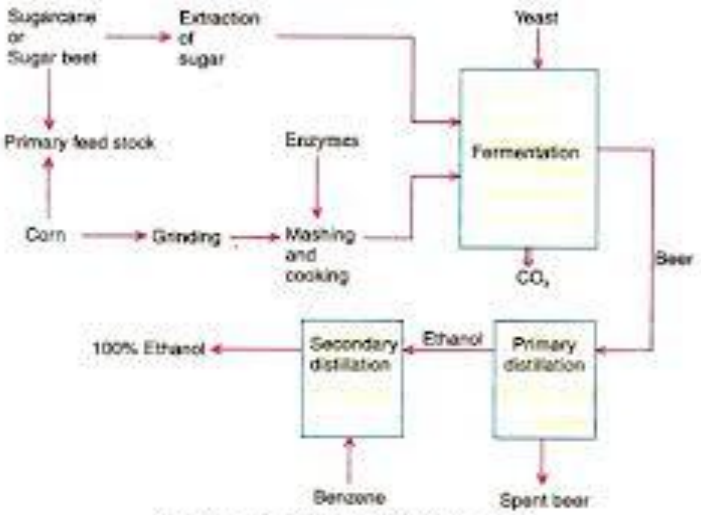


Fig. 20.6: Ethanol production from molasses.

Distillation

Distillation is one of the steps of the purifications. Distillation is the method to separate two liquid utilizing their different boiling points. After fermentation the fermented liquor contains alcohol as well as low boiling point volatile compounds such as acetaldehydes, esters and the higher boiling, fuel oils. The alcohol is obtained by several operations. First, steam is passed through the liquor. The result is a dilute alcohol solution which still contains part of the undesirable volatile compounds. Secondly, the dilute alcohol solution is passed into the center of a multi-plate aldehyde column in which the following fractions are separated: esters and aldehydes, fuel oil, water, and an ethanol solution containing about 25% ethanol. Thirdly, the dilute alcohol solution is passed into a rectifying column distils off at 95.6% alcohol concentration. The maximum alcohol concentration of 96.5% is obtained by azeotropic distillation. The principle of this method is to add an organic solvent which will form a ternary (three-membered) azeotrope with most of the water, but with only a small proportion of the alcohol. Benzene, carbon tetrachloride, chloroform and cyclohezane may be used, but in practice, benzene is used. In practice,four columns are usually used. The first and second columns remove aldehydes and fuel oils, respectively, while the last two towers are for the concentration of the alcohol.

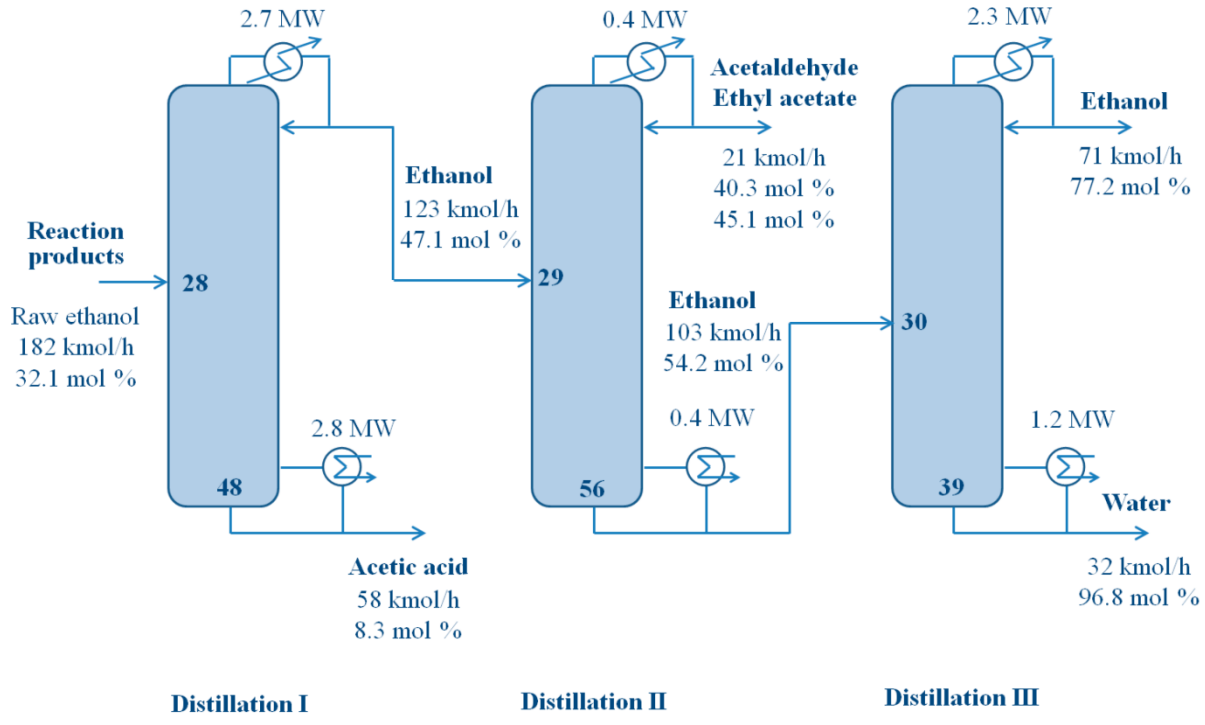


Figure. Schematic diagram of distillation columns in the conventional process for the ethanol concentration. The numbers in the columns indicate tray numbers, counted from the top.

Some Developments in Alcohol Production

(1) Developments of new strains of yeast of Saccharomyces uvarum able to ferment sugar rapidly, to tolerate high alcohol concentrations, flocculate rapidly.

(2) The use of continuous fermentation with recycle using the rapidly flocculating yeasts.

(3) Continuous vacuum fermentation in which alcohol is continuously evaporated under low pressure from the fermentation broth.

(4) The use of Zymomonas mobilis, a Gram-negative bacterium which is found in some tropical alcoholic beverages. The advantages claimed for the use of Zymomonas are the following:

(a) Higher specific rates of glucose uptake and ethanol production than reported for yeasts.

(b) Higher ethanol yields and lower biomass than with yeasts.

(c) Ethanol tolerance is at least as high or even higher [up to 16% (v/v)] in some strains of the bacterium than with yeast.

(d) Zymomonas also tolerates high glucose concentration and many cultures grow in sugar solutions of up to 40% (w/v) glucose which should lead to high ethanol production.

(e) Zymomonas grows anaerobically and, unlike yeasts, does not require the controlled addition of oxygen for viability at the high cell concentrations used in cell recycle. (f) The many techniques for genetic engineering already worked out in bacteria can be easily applied to Zymomonas for greater productivity.

Acetic Acid

Introduction

The bridge between chemistry and the day to day human life is always growing wider and stronger, and acetic acid is one of the perfect examples. Acetic acid is a clear liquid with a pungent odour, sharp taste, melting point of 16.73°C and boils at 117.9°C. Acetic acid, traditionally known as ‘vinegar’ is widely used as a food preservative, first discovered (c. 5000 BC) when unattended grape juice turned in wine. A famous physician Hippocrates II (c. 420 BC) used acetic acid to clean the wounds. With direct and indirect applications of acetic acid, it has diversified into several chemical sectors such as food, pharma, chemical, textile, polymer, medicinal, cosmetics etc. Since then, acetic acid is proven to be a multi-application chemical building block resulting in ever-increasing demand. The production of acetic acid is expected to reach 18 million ton with an average growth of 5 % per year. The overall routes for production and the applications of acetic acid are shown. Currently, the manufacturing demand is fulfilled via two main production routes, which are chemical and fermentative.

Amongst the chemical manufacturing processes, the key processes are Cavita process (carbonylation of methanol), oxidation of aldehyde and oxidation of ethylene. The major players are BP chemicals and BASF which follow carbonylation route. The major consumption of acetic acid mainly comes from the preparation of vinyl acetate monomer (VAM), acetic anhydride, C1-C4 acetates and it is used as a solvent in synthesis of terephthalic acid (PET). VAM is a one of the main ingredients used in polymer industry with application in as emulsifier, resins, as intermediate in surface coating agent, acrylic fiber and polymer wires. It is also used in textile industry to generate synthetic fibers as a result of condensation rection. The other condensation reaction of acetic acid produces acetic anhydride used as typical acetylation agent, which is subsequently utilized to produce cellulose acetate, used in synthetic textiles and for silver-based photographic films. Most derived esters of acetic acid are ethyl acetate, n-butyl acetate, isobutyl acetate, and propyl acetate which are frequently used as solvents for inks, paints and coatings. Glacial acetic acid is an excellent polar protic solvent which is frequently used as a solvent for recrystallization to purify organic compounds. Several researchers are working on developing a sustainable process with the simple design to produce acetic acid that meets current demand. Several homogeneous, as well as heterogeneous catalytic systems, are reported for the production of acetic with carbonylation process.

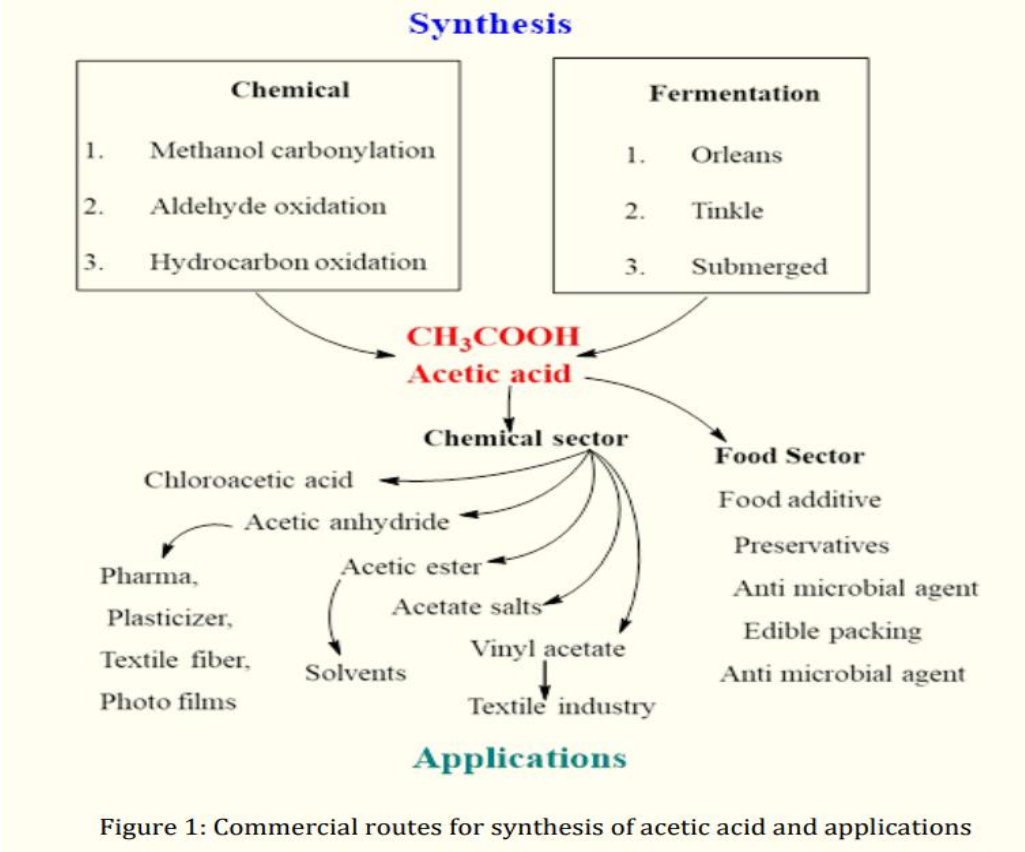


Figure 1: Commercial routes for synthesis of acetic acid and applications

Acetic acid produced via fermentation route is mainly utilized in the food industry in the form of vinegar. Use of vinegar is more diversified these days coming with more innovative ways to adjust and suit the current lifestyle and food culture. The different concentrations of acetic acid are used to sharpen the taste of food with a longer shelf life period and as a food preservative. Some new application has also come such as edible and non-edible antimicrobial coating. This chapter reviews the current commercial processes for the synthesis of acetic acid to meet an ever-increasing global demand. The chapter also gives insight into the pros and cons associated with the process available and then how should we design a sustainable strategy to develop a simple commercial process. Further, the state of art to produce vinegar is discussed with exploitation as a multiapplication tool in the modern food industry.

Production of acetic acid

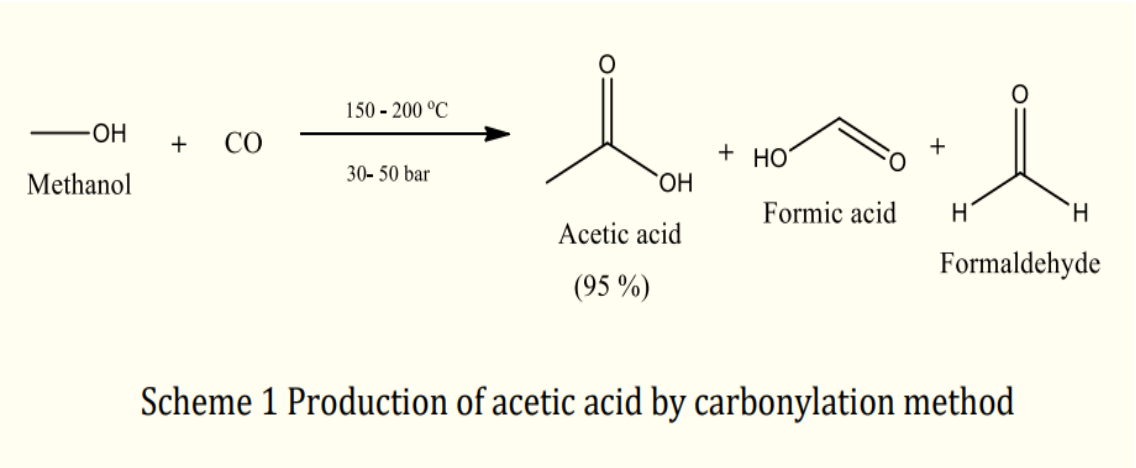
Acetic acid is mainly produced via chemical route which involves homogeneous as well as heterogeneous catalytic methods. The carbonylation of methanol via Monsanto process is the most adapted route, which further evolved as Cavita process with a choice of catalysts and process intensification. In the recent decade, the fermentative approach has also gained attention, however the commercial approach is not established yet. The current trends in sustainable manufacturing demand an urgent paradigm shift to develop and pursue more sustainable routes to reduce environmental burden. An approach is also made with the development of membrane-based technology which offers a very simple design with eco-friendly production.

Conventional process

I Methanol carbonylation process:

Carbonylation process is a most employed commercial route for synthesis of acetic acid, also known as Monsanto process (Scheme 1). Methanol and carbon monoxide are reacted in liquid phase in the presence of rhodium (Rh)-based catalyst at 150-200 oC temperature and 30-50 bar pressure to produce acetic acid with 95% selectivity and 5 % side products such as formic acid and formaldehyde. Hydrogen iodide is used as an alkali promoter in this process. The reaction proceeds in liquid phase with methyl acetate as solvent using homogeneous catalyst. The controlled amount of water is required for the reaction which is generated in situ by reaction of methanol with hydrogen iodide. The rate of reaction in the Monsanto process depends on the concentration of water. CO2, H2 and methanol are obtained as byproducts in the reaction.

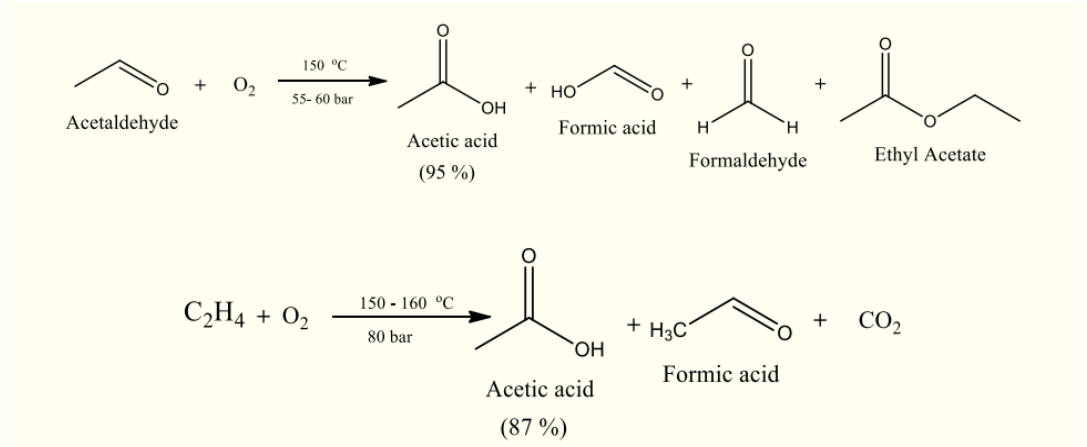
The generated methanol in the reaction is recycled. The process has evolved with the time and different strategies were adapted to separate pure acetic acid from a mixture of water and byproducts. This process was modified by BP chemicals replacing rhodium-based catalyst with Iridium (Ir) catalyst known as Cavita process. The choice of Ir as a coordination metal is relatively more economic process than rhodium. The use of an iridium catalyst improves the overall rate of reaction. The safety and the environmental hazards arising from the current methods are a serious concern. Acetic acid is highly corrosive, and the production processes need to be more sustainable and environmentally benign by reducing the amount of energy required in production and subsequent separation technologies as well as using heterogeneous catalysts. The Japanese firm, Chiyodo developed a heterogeneous Rh catalysed process wherein, Rh metal was immobilized on the vinyl-pyridine resin. The use of heterogeneous catalyst prevails the loss of catalyst in the liquid phase and facilitates easy separation from the reaction mixture. The amount of water used in the reaction is very low and thus the separation of water from acetic acid is more energy-efficient compared to the other processes mentioned.



Scheme 1 Production of acetic acid by carbonylation method

II Acetaldehyde oxidation process

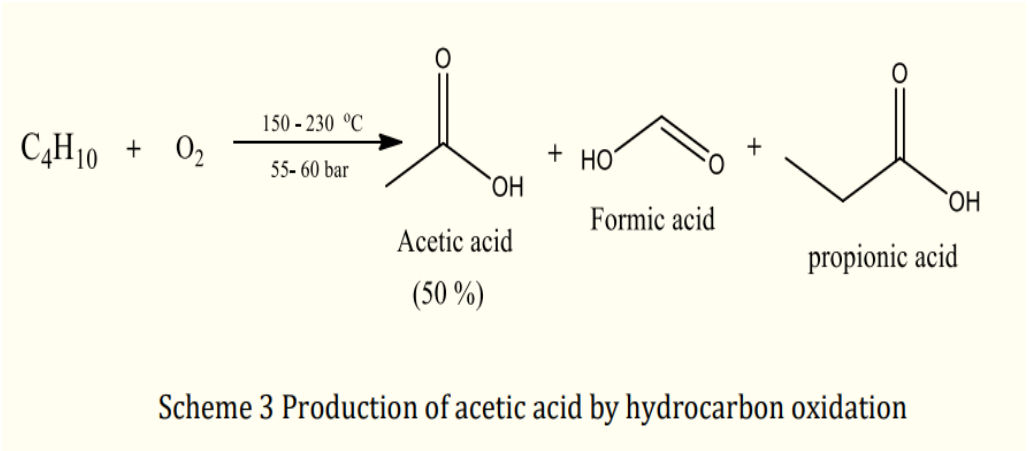
Acetaldehyde oxidation was the predominant process followed for the synthesis of acetic acid. Wherein, acetaldehyde is first prepared by oxidation of ethylene using palladium and copper chloride and it was further oxidized to form acetic acid (Scheme 2). The same process is reported using cobalt and chromium-based catalyst at 55 bar pressure and 150 °C temperature. The one-step process for conversion of ethylene to acetic acid is also practiced using lead and lead-platinum based catalyst at high pressure compared to the acetaldehyde oxidation process with a low yield of acetic acid.



Scheme 2 Production of acetic acid by acetaldehyde oxidation

III Hydrocarbon oxidation process

Hydrocarbons derived from petroleum stock such as butane and naphtha are utilized to generate acetic acid using cobalt acetate and chromium acetate catalyst (Scheme 3). The reaction proceeds at a comparatively higher temperature range (150-230 °C) and pressure (50-60 bar). The process involves petroleum feedstock which contains hydrocarbon mixture which leads to the formation of other byproducts such as acetone, formic acid, propionic acid along with acetic acid. Thus, this process fails to give pure acetic acid. This process is more suitable for manufacturing a mixture of volatile fatty acids.



Scheme 3 Production of acetic acid by hydrocarbon oxidation

Fermentation route

Fermentative route is mostly adapted for the generation of food-grade acetic acid i.e. vinegar. This process mainly involves the use of renewable carbon resources such as apple, grape, pears, honey, cane, coconut, date, syrup cereals, hydrolyzed starch, beer and vine. The fermentation process is mainly divided into two steps: the treatment with yeast followed by acetic acid bacteria (AAB). Commercial production of vinegar is done via oxidative fermentation using AAB. Acetobacter and Gluconacetobacter are most used species among ten classified genera. Acetobacter pasteurianus is traditionally used for commercial production of vinegar with concentration not exceeding 6 % (v/v). Whereas, Gluconacetobacter europaeus is utilized to produce high concentration vinegar (10 % v/v). The price of the vinegar varies with the kind of source is used and the region where it is generated.

I) Orleans method:

This method is well established, traditional and preferred for low volume production of acetic acid. Derived from the French word Orléans, wooden barrels are used to ferment the feed in this process. This method is followed to prepare exotic brands of vinegar in different regions of the world with specific raw material available in the specific season. The traditional balsamic vinegar produced in different parts of the world such as sherry from Spain, oxos from Greece and Modena in Italy.

II) Trickling process:

This process was developed to overcome the slow rate of acetification in Orleans process. The process intensification was done improve the acetic acid bacteria and substrate interaction. The alcoholic substrate was sprayed over the fermentation in continuous loop to achieve the desired concentration of acetic acid. The heat of the reaction was controlled by passing the air through the system. The process has drawback of accumulating gelatinous material on the surface the membrane which reduces the rate of reaction over the period.

III) The continuous submerged process: This modern fermentation method is followed to produce vinegar in masses. This is the most widely method and has a high yield along with the fast rate of oxidation as compared to the previous method. This method is 30 times faster than the Orleans method with higher efficiency for production of acetic acid. This process requires comparatively small space with higher yields.

The Fringe fermenter is used for this process to increase the rate of the acetification. The yield of acetic acid is 98 %. The pure substrates are required to achieve the high quality of acetic acid. This fermentation process is much economical, simple design with easy process control.

The Fermentation process for acetic acid is economically feasible with comparatively simple operations. The application of this process is very limited to the present global demand. Whereas, the conventional process involves several steps such as fractional distillation, condensation and crystallization, which add to the high machinery cost. The operating conditions are harsh considering the process temperature and pressure along with the corrosive nature of acetic acid. The purification of acetic acid from water is a multi-step process which consumes a high amount of energy which makes overall process complex and critical. In addition to this, the process requires huge manpower with stringent safety protocols and norms.

Need for development of Novel sustainable technologies

Looking at the ever-increasing threats of global warming and ever-increasing global demand of acetic leads into it is an urgent need to develop a novel technological approach and sustainable feedstock for the generation of acetic acid. Even though many process and technological development are reported recently, they fail to sustain the production cost to profit margins. The separation of acetic acid remains the key issue to overcome the economical and energy consumption barriers. The different operations such as distillation, evaporation, absorption, filtration crystallization and alkali neutralization are time and energy consuming. Even though these processes involve multiple steps, the ever-growing demand forces to follow this path. On the other hand, fermentation process is reliable but cannot match the scale of current demand. Thus, the development of novel route for generation or process intensification in separation can drastically reduce the overall production cost of acetic acid. Utilization of CO and CO2 as feed stock generated from natural gas can offer long term sustainability of acetic acid production. This technology offers high purity of acetic acid with eco-friendly production. Furthermore, membrane-based separation processes can provide efficient way to produce acetic acid. The pathways are discussed briefly.

CO and CO_2 as valuable feedstock:

Utilization of CO_2 and syngas can offer sustainable alternatives to produce acetic acid. BP has announced the breakthrough process wherein, acetic acid will be manufactured from syngas as a feedstock derived from natural gas.

This will give an alternative to SaaBre process which produces acetic acid in three integrated steps. The production of acetic acid from syngas will avoid the purification of CO and purchase of methanol.

Though the technology is not fully developed, it provides better alternatives in terms of sustainability. Similarly, acetic acid can also produce via CO2 and H2 to produce methanol followed by subsequent carbonylation step. This route gives liberty to utilize CO2 as value added feedstock

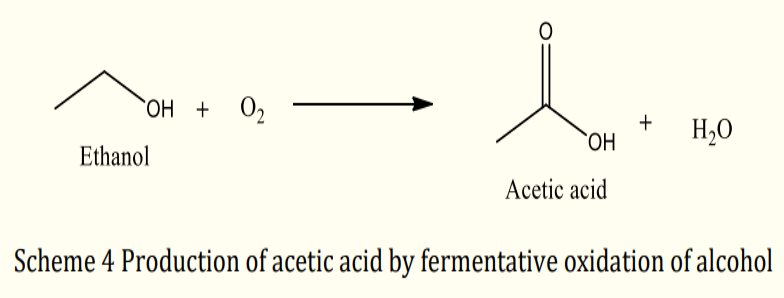
Membrane based technologies: The membrane technology can offer the separation of liquid, vapor and gas selectively with controlled mass transfer rates. These processes are easy to operate and simple to design. The technology can offer development on energy intensification. Several types of processes are reported based on the pore size of the membrane for separation of different components. These are namely microfiltration, ultrafiltration and nanofiltration membrane. The operating pressure (varying from 1 – 20 bar) of the system varies according the pore size of the membrane are used. The reverse osmosis is another membrane technology with non-porous membrane. This process operates at pressure more than 20 bar. The membrane technologies collectively can be applied in downstream processing for separation of acetic acid in chemical process as well as fermentation processes. The combination of fermenter with acetic acid permeable membrane can help in separation of acetic acid to avoid the self-inhibition.

Application of acetic acid in food industry

Direct applications of acetic acid are reported from ancient times. It was used as a medicine and food preservative. Over the period of applications of acetic are diversifies as per the demands of modern life. Using the different concentrations, it is utilized as Food Additives, Food Preservation, Antimicrobial Agent, Acidulant, Flavor and Taste enhancer, Edible Packaging material, artificial food ripening agent etc. Some of the applications such as acidulent and as acetification agents are described in detail here.

Acetification

Acetification is simply bacterial oxidation of ethanol to produce acetic acid and water. The process is also termed as oxidative fermentation. The rate of the reaction in acetification mainly depend on type of micro-organism used to catalyze and the concentration of available oxygen in the media



Scheme 4 Production of acetic acid by fermentative oxidation of alcohol

There are different types of microorganisms which occur naturally in food which are responsible for the different natural process such as acetification, alcoholism, proteolysis and enzymatic reactions which alter the natural condition of the food. This bioprocess technology is studied and systematically utilized to improve the quality of food s in terms of texture, taste, mouthfeel, colour and prolonged shelf life. The overall concept has grown into generating different types of food and beverages produces in a cheap and sustainable way.

Acetification of different food categories using acetic acid bacteria (AAB) has led to the production of several food products. AAB are naturally found on fruits, flowers and plants which naturally react and convert carbohydrates sugars into organic acids in presence of oxygen. The same concept is biotechnologically utilized to prepare a diverse variety of food and beverages.

Flavoring agent:

The different parts of the world have utilized the acetification process to generate a variety of foods and beverages. The famous Lambic beer is produced from malted barley, aged dry hops and unmalted wheat. The different AAB and yeast are responsible for the generation of this beer which is matured for over the period of three years. The typical acidic flavour of the beer is achieved with help AAB together with lactic acid. The sparkling water is another famous example which gives typical acidic and fruity flavour vis fermentation of water and natural sucrose. Water kafir is one of the examples of such type. Kombucha is another type of beverages produced by oxidative fermentation. It is prepared from Kombucha (tea fungus), water and sugar. Similarly, Cocoa is fermented from cocoa beans with the help of AAB and yeast which is used as raw material for chocolate production.

Acidulent

Acidulants are essential ingredients or additives which are generally used to improve the taste of food and make it sharper. There are naturally occurring acidulants such as acetic acid, citric acid, malic acid, fumaric acid, lactic acid, tartaric acid, succinic acid, phosphoric acid etc. having different taste profiles. Many fruits such as orange, lemon, apples, tomatoes, and yogurt contain natural acids with the most common example as citric acid. Citric acid comes with lemon flavour, acetic acid with strong familiar vinegar flavour, tartaric acid gives sharp taste and lactic acid is with a smooth taste. Apart from taste enhancement, acidulants also act as a food preservative.

The choice of the acidulent is usually made on their characteristic flavour and the physical state and solubility. Some food formulations require solid acidulants. In general, inorganic acids such as sulphuric acid, phosphoric acid, monosodium orthophosphate and diphosphates are used as dry acidulants in controlled concentrations. The composition of the acidulants is based on their selection and different concentrations calculated by total titratable acid. Acetic acid is mainly used in the form of vinegar with the pungent smell. As it appears in the liquid state, it is used as a preservative in pickles. It is also used in the manufacture of cheese to improve the shelf life period, good mouthfeel and taste.

Edible packing:

Acidulants are also used as food coating; it may be edible or non-edible to prevent it from contamination with the surrounding environment, to protect it from bacterial infection and to improve the shelf life of the food. These films are easily biodegradable. The water-soluble non-edible coating is used for the packing of food [16]. The edible coating is used for breath freshening agent, in drug delivery and as flavour. Acetic acid is used in edible films to enhance sour flavour. Various compositions of acetic acid are used to develop antimicrobial food coating to stop the outgrowth of bacterial and fungal cells. It is also used in meat coating and preservation of meat products. The chitosan-based edible food coatings along with aqueous acetic acid is used to enhance anti-listerial activity.

Antibacterial agent:

Acetic acid is commonly used in medicine since ancient times. The low concentrations (3 %) of acetic acid can be used as a local antiseptic against various micro-organisms. Acetic acid is always considered as an alternative It can be utilized as in vitro antimicrobial agent combined with other antiseptics. Acetic acid covers the vide range of spectrum with Gram-positive as well as Gramnegative bacteria.

Penicillin

Penicillin a broad-spectrum antibiotic belongs to β-lactam group and is active against many Gram positive bacteria including actinomycetes. This antibiotic was first discovered by Alexander Fleming in 1929 and the producing organism associated with its production was Penicillium notatum. His findings remained unnoticed until 1940 when Sir Howard Florey and E B Chain at Oxford University studied Fleming's observations systematically, and used this antibiotic to treat mice injected with lethal dose of Streptococcus. A patient named Albert Alexander suffering from septicaemia caused by streptococcal and staphylococcal infection was the first person treated with this antibiotic in 1941. In the beginning the response was good but later he died because of insufficient amount of penicillin required for the treatment. Both of the researchers went to US in 1941, where the scaling up of the production process started. During that time US entered in the World War II, and the penicillin production programme was declared 'top secret' by the War Production Board of US as this antibiotic had great potential for treating the bacterial infection occurring to the war injured soldiers. After end of the World War II penicillin production started in other countries, and Fleming, Florey and Chain were awarded with Noble Prize for Medicine and Physiology in 1945 for this great scientific discovery. Till date penicillins and their derivatives share the major antibiotic market.

The basic structure of the penicillin is 6-amonpenicillinic acid (6-APA) which consists of a thiazolidine ring with a condensed β-lactam ring (Fig 13). Depending upon the acyl moiety at position 6 there are various penicillins. The acyl group to be attached to the ring is either added in the growth medium producing biosynthetic penicillins or it can be attached chemically to 6- APA obtained by deacetylation of penicillin through acylases forming semisynthetic penicillins. The penicillin produced without addition of any side-chain precursors are referred as natural penicillins. Penicillin G, penicillin V are the important natural penicillins.

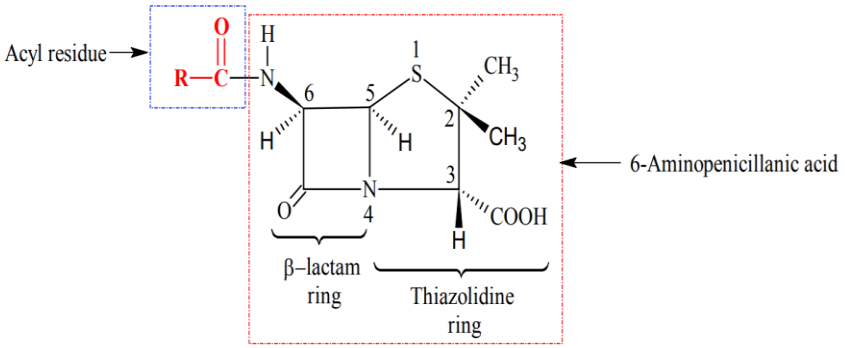


Fig. 13 : Chemical structure of penicillin

Biosynthesis of penicillin

Three amino acids namely L-α-aminoadipic acid, L-cysteine, L-valine are involved in the synthesis of penicillin (Fig 14). These three amino acids are converted into tripeptide L-αaminoadipoyl-L-cysteinyl-L-valine (ACV) through nonribosomal process catalysed by ACV synthetase (ACVS). ACV is then cyclized to form a bicyclic isopenicillin N (IPN) catalysed by non-haem iron (II) dependant oxidase isopenicillin N synthase (IPNS). The penicillin 32 transacylase converts α-aminoadipoyl side chain to one of many other alternatives, giving rise to myriad of natural and non-natural penicillins.

Mechanism of action

Penicillin inhibits the cell wall synthesis in growing bacterial cells by combining with the socalled penicillin-binding protein (PBP) of the bacterial cell wall involved in the transpeptidation reaction (cross linking) of the peptide side chains of the adjacent peptidoglycan in the bacterial cell wall. Due to the defective cell wall, the bacteria are unable to withstand the osmotic shocks and are ultimately get lysed.

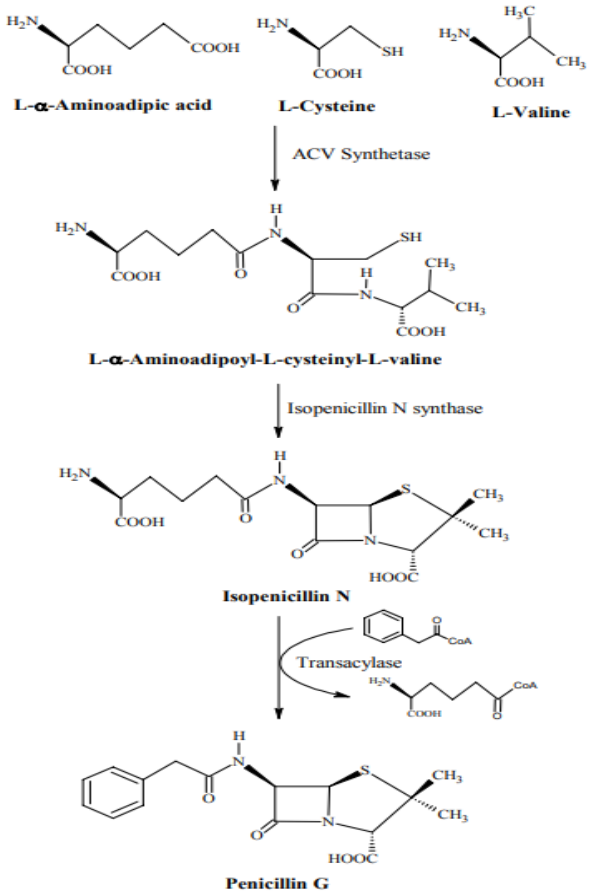


Fig. 14: Biosynthesis of penicillin

Strains and production of penicillin

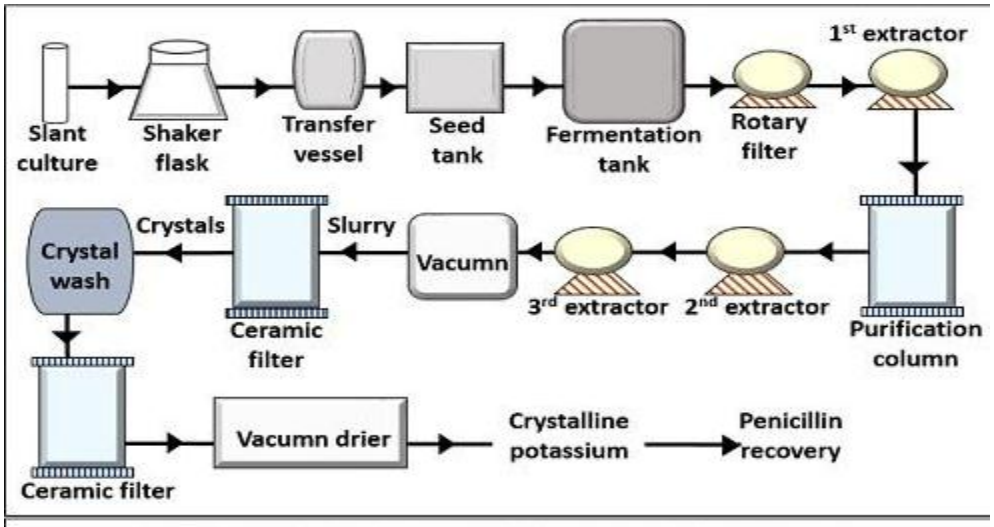
The strain of Penicillium notatum discovered by Fleming had low productivity (2 IU/ml). Later in the year 1945 Raper and Alexander found a new strain Penicillium chrysogenum having higher yield of penicillin. Further improvement was achieved by inducing mutations via UV irridaition and strain Wis Q 176 was isolated. Presently the strains have been improved much to produce 15000 IU/ml of penicillin during fermentation.

The media used for the production of penicillin contains cornsteep liquor solids 3.5%, lactose 3.5%, glucose 1%, calcium carbonate 1%, potassium dihydrogen phosphate 0.4%, edible oil 0.25% and penicillin precursors.

Submerged fermentation processes in 40,000-200,000 liter fermenters with high aeration and agitation is carried out. The pH of the media is maintained around 5.5-6.0 and temperature about 25-27 ºC. Lyophilized spores are used as inoculum to a concentration of 5 x 103 /ml medium and development of loose mycelial pellets are required for better production of penicillin. The penicillin fermentation process can be divided into two phases i.e. first is the growth phase and the second is the penicillin production phase. The growth phase lasts for about 40 h during which the cell mass increases very rapidly (doubling time 6 h) and oxygen demand is very high. After this phase the penicillin production phase starts in which the biomas production is greatly reduced (specific growth rate, $\mu = 0.01$) and rate of penicillin production increases. Various media component are fed during this phase to extend the penicillin production for 120-180 h.

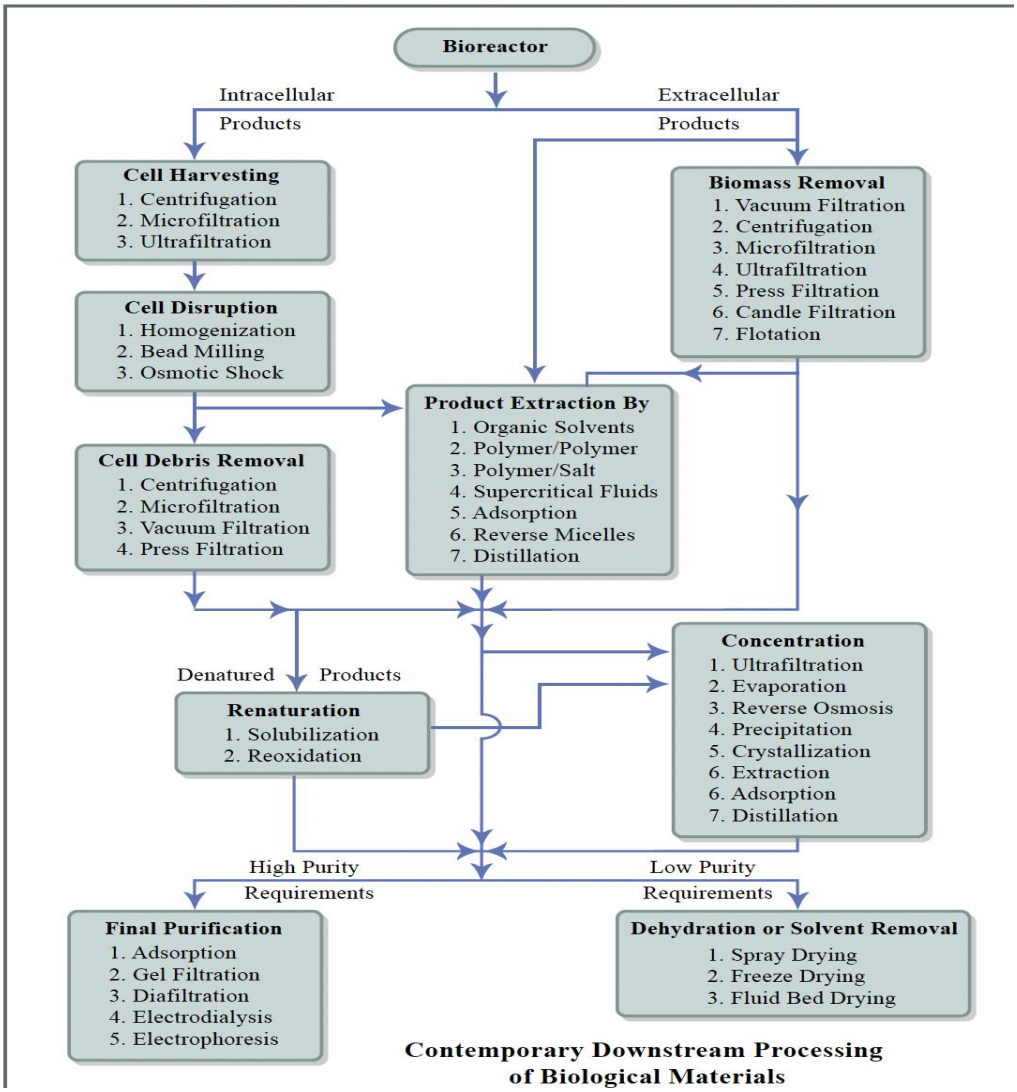
Recovery of penicillin

In the downstream processing of penicillin G, the fermentation broth is first chilled and passed through rotary filter to remove biomass. The filtrate is then acidified and the penicillin is extracted with butyl acetate. Penicillin is extracted from solvent (butyl acetate) into aqueous buffer with pH 7.0. The aqueous fraction is acidified to pH 2.0-2.5 with H2SO4 and re-extracted into butyl acetate. Potassium acetate is added to the extract which causes the formation of penicillin GK+ salt crystals via a number of back-extraction and crystallization steps. The salt so formed is washed with acetone by centrifugation and dried under vaccum.



PRODUCTION PROCESS OF PENICILLIN

WHEN SELECTING UNIT OPERATIONS THERE ARE CHOICE AND DECISIONS MUST BE MADE



Contemporary Downstream Processing of Biological Materials

Vitamin B_{12} (Cyanocobalamin)

In 1920 G H Whipple was conducting an experiment to induce anemia in dogs by bleeding them and observed that ingestion of raw liver by the dogs cured their anemia. Whipple did not applied his finding to cure human pernicious anemia. Later in 1926, G R Minot and W B Murphy based 39 on the studies of Whipple used liver extracts to cure human pernicious anemia, and all these three persons shared Nobel Prize in medicine in 1934. The anti pernicious anemia factor from the liver was independently isolated and crystallized by Ricke and coworkers at Merck Laboratories and Smith and Parker at Glaxo Laboratories and named it as cyanocobalamin (vitamin B12). In nature this vitamin is exclusively synthesized by certain prokaryotic microorganisms and it is present at ppm level as adenosyl- or methylcobalamin in every animal tissue (1 ppm in liver).

A specific B12 coenzyme synthetase converts vitamin B12 (Co+2) to vitamin B12 (Co+1) which forms coenzyme of various enzymes catalyzing cleavage of carbon-carbon, carbon-oxygen and carbon-nitrogen bonds and transfer of methyl group. In humans, the vitamin is required in trace amounts (approximately 1 µg/day) to assist the actions of two important enzymes i.e. methionine synthase and (R)-methylmalonyl-CoA mutase. The other biochemical reaction involving vitamin B12 occur in bacteria, e.g., methanogenesis. Vitamin B12 along with folic acid participates in the shrinking of newly synthesized RBC making them less fragile.

The deficiency of vitamin B12 results in low RBC which are larger in size and get damaged quickly. Nerve damage also occurs in the hands and feet and leads to a feeling of pins and needles and numbness. The animals obtain vitamin B12 from the food or by absorption of vitamin B12 produced by enteric microorganisms. Humans are solely depended on dietary vitamin B12 because of non-assimilation of the vitamin B12 produced by the microorganism present in large intestinal tract.

Annual production of vitamin B12 using fermentation process is more than 10 tonnes from a number of bacterial species. The discovery of vitamin B12 in the spent medium of streptomycin and other antibiotic fermentation replaced the use of beef liver for its production. Around 1950 vitamin B12 was isolated from antibiotics fermentation broths and dried sewage residue of activated sludge process. Presently this vitamin is being produced by fermentation process using improved bacterial strains. The major use of the vitamin B12 is animal feed supplementation in the pharmaceuticals for curing pernicious anemia.

Vitamin B12 is chemically related to cobamides consisting of a cobalt porphyrin nucleus to which ribose is linked via phosphate ester bond.

In the porphyrin, tetrapyrole ring also referred as corrin ring lacks the methane bridge between rings A and D. Five of the six atoms attached to the cobalt are nitrogen, four of which form the tetrapyrole ring and the fifth one is from the 5,6- dimethylbenzimidazole attached to C-1 of the ribose (Fig 18). The sixth ligand could either be deoxyadenosine, cyanide or a methyl group, forming adenosylcobalamin (coenzyme B12), cyanocobalamin (vitamin B12) and methyl cobalamin respectively.

Biosynthesis

The chronology of the discoveries along the pathway of vitamin B12 biosynthesis is reviewed by Scott (2003). Two pathways for the synthesis of vitamin B12 have been suggested i.e. one aerobic and another anaerobic which mainly differ in the ring contraction mechanisms converting porphyrin to corrin. The major difference between the two is the timing of cobalt insertion. In anaerobes, cobalt is inserted early in the pathway into precorrin-2 whereas aerobes insert cobalt later in hydrogenobyrinic acid a, c-diamide. All seven methyl groups to the corrin ring are 40 donated by the alkylating agent S-adenosylmethionine (SAM). Some of the techniques involved in discovering the biosynthetic pathway of vitamin B12 included NMR, gene mutation and cloning. The biosynthesis of vitamin B12 runs parallel with the biosynthesis of porphyrins and chlorophyll up to the formation of uroporphyrinogen III by succinate-glycine pathway. The uroporphyrinogen III (Urigen III) synthesis starts with the enzyme δ-aminolevulininc acid synthetase (pyridoxal phosphate–dependent) catalyzing the decarboxylation of glycine and then condensing it with succinyl-CoA to produce δ-aminolevulinic acid (ALA). The steps and enzymes involved in the biosynthesis of this vitamin are summarized in Fig. 19.

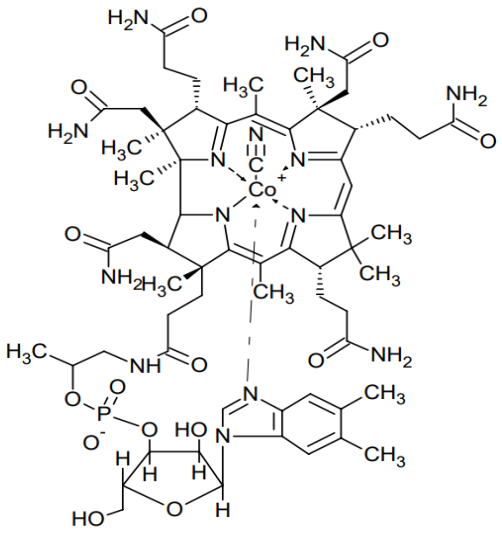


Fig 18: Chemical structure of cyanocobalamin

Production processes

The concentration of vitamin B12 is very low in animal tissue and could not be used for commercial production. The activated sludge from the sewage treatment contains 4-10 mg B12/kg has limitations in the separation of various B12 froms present in it. The chemical synthesis of this vitamin involves 70 steps which is impractical to apply at commercial scale. Vitamin B12 was first commercially produced as by product of various Streptomyces fermentations for the production of the antibiotics (streptomycin, chloramphenicol, neomysin) with an yield of about 1 mg/L. The commercial production of vitamin B12 is currently based on fermentation processes.

Bacillus megaterium (0.45 mg/L), *Butyribacterium rettgeri* (5 mg/L), *Streptomyces olivaceus* (3.3 mg/L), *Micromonospora* sp. (11.5 mg/L), *Klebsiella pneumoniae* (0.2 mg/L) have been reported to produce vitamin B12. In most of the fermentation processes glucose is used as a carbon source. High yields of vitamin B12 from *Propionibacterium freudenreichii* (19 mg/L) and *P. shermanii* (30-40 mg/L) have also been obtained. A strain of *Pseudomonas denitrificans* in a process based on sugarcane molasses yielded 60 mg/L of vitamin B12. *Rhodopseudomonas protamicus* developed by protoplast fusion of *Protaminobacter ruber* and *Rhodopseudomonas spheroides* produces 135 mg/L vitamin B12 in the fermentation broth containing glucose as carbon.

A number of new strains able to utilize alcohols and hydrocarbons as carbon source have very good potential to synthesize vitamin B12. Methanol as carbon source has higher yields in comparison to higher alcohol e.g *Methanosarcina barkeri* produces 42 mg/L vitamin B12 when methanol is added in fed batch mode.

Propionibacterium freudenreichii ATCC 6207 and P. shermanii ATCC 13673, and some mutants are used for vitamin B12 production in a two-stage batch fermentation process to facilitate overproduction. The first stage is the anaerobic one and lasts for 2-4 days during which B12 precursor 5´-deoxyadenosylcobinamide accumulates and no vitamin B12 is synthesized 42 preventing its repressive effect on the biosynthesis. The second stage is aerobic one (3-4 days) in which 5,6-dimethylbenimidazole synthesis takes place or it is added to the medium, which finally combines with 5´-deoxyadenosylcobinamide to produce vitamin B12 (5´-deoxyadenosylcobalamin).

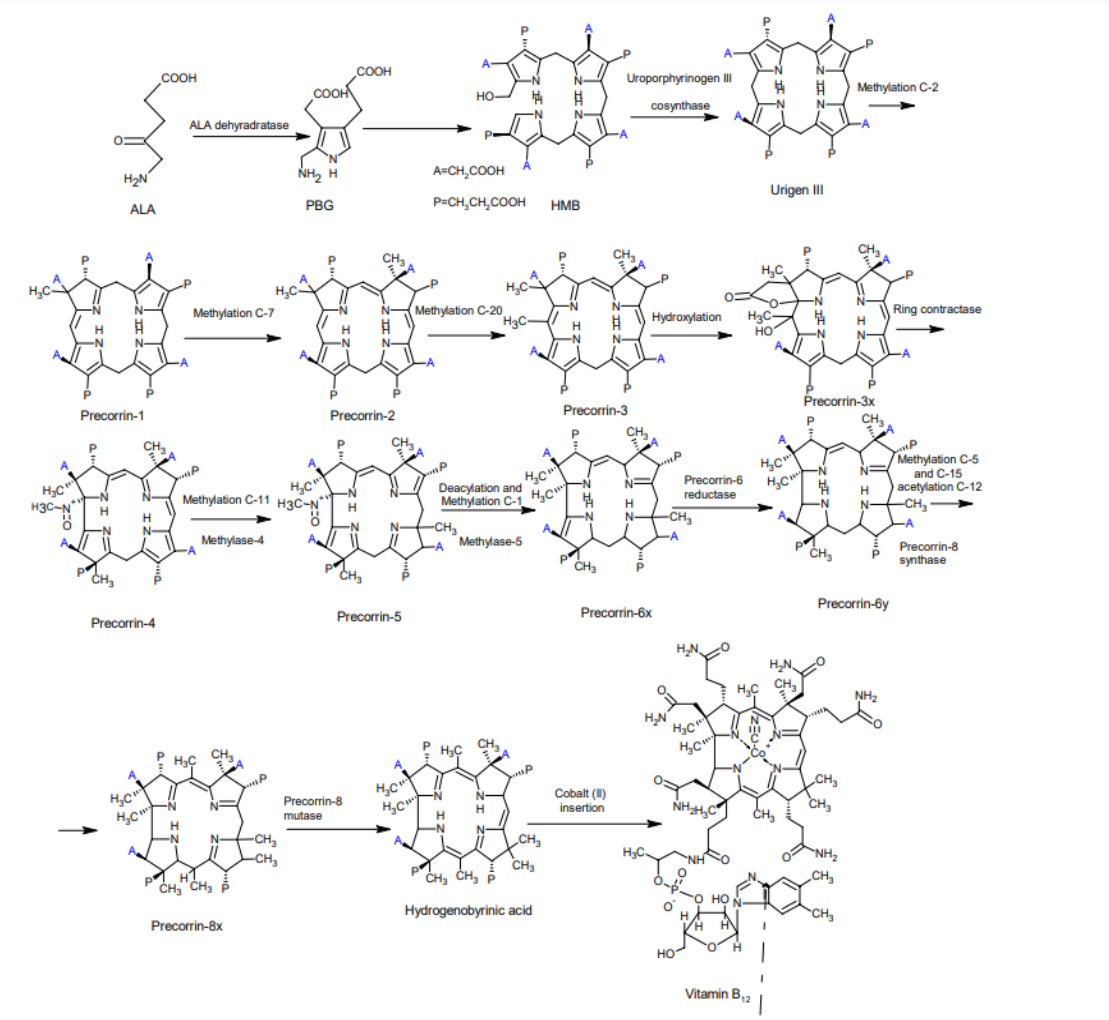


Fig. 19: Pathway for the biosynthesis of Vitamin B_{12} in aerobic bacteria
(Source : Scoot, 2003)

Fermentation using *Propionibacterium freudenreichii*

The fermentation medium is supplemented with cobalt (10-100 mg/L). Alternative to batch fermentation, the two stages of vitamin B12 fermentations can be performed in a continuous mode with two tanks in a cascade manner.

Fermentation using *Pseudomonas denitrificans*

Pseudomonas denitrificans is the most productive species among the so far reported pseudomonads producing vitamin B12. The fermentation medium contains 10% sugar beet molasses, 0.2% yeast extract, 0.5% (NH4)2HPO4, 0.3% MgSO4·7H2O, 0.02% MnSO4·H2O, 0.0188% Co(NO)3·6H2O, 0.0025% 5,6-dimethylbenzimidazole, 0.002% ZnSO4·7H2O, 0.0005% Na2MO4·2H2O, and fermentation is carried out at 7.4 pH, 420 rpm, 1 vvm aeration and 29 ºC for 90 h (Merck and Co., Inc., 1971). The strain development studies have increased the vitamin B12 yield from 0.6 mg/L to 60 mg/L.

Most of the cobalamin synthesized by the microorganism is secreted in the broth but 20% remains bound to cells. The fermentation broth is heated for 10-30 min at 80-120 ºC, pH 6.5-8.5 to solubilize all the vitamin B12. The heated broth is treated with cyanide or thiocyanate to form cyanocobalamin which is separated by cation exchanger (Amberlite IRC 50). It is either extracted with organic solvent (phenol or cresol or in a mixture of benzene, butanol, carbon tetrachloride, or chloroform) or precipitated or crystallized by evaporating the diluents such as cresol or tannic acid containing vitamin B12. In total 98% of the vitamin B12 is recovered from the fermentation broth. Vitamin B12 having 80% purity is used as feed additive and high purity preparations are used in pharmaceutical formulations.

Amylases

Higher plants store carbohydrates in the form of starch (granules) which is composed of 20-30% amylose (linear polymer of 500-20,000 α-1,4 linked D-glucose units) and 70-80% amylopectin (branched polymer formed by joining of linear polymer of 24-30 α-1,4 linked D-glucose units by α-1,6 glycosidic bond). Starch hydrolyzing enzymes are referred as amylases, and are mainly used in the production of sweeteners for the food industry. Enzymatic hydrolysis of starch first produces short-chain polymers of glucose called dextrins, then the disaccharide maltose, and finally glucose. Starch saccharification process involves use of α-amylases, β-amylases, glucoamylases, pullulanases and isoamylases glucose isomerases.

The commercially important amylases of microbial origin that split α-1, 4 and/or α-1, 6 bonds in starch molecule have been classified into six groups and the specific glycosidic bond hydrolysed by amylases is depicted in.

α-Amylases

α-Amylases (1,4- α-glucan-glucanohydrolases) are extracellular enzymes which hydrolyze α-1,4-glycosidic bonds present in the interior of starch and thus are endoacting enzymes. α-Amylases are produced by many bacteria and fungi and are classified on the basis of their starch-liquefying and/or saccharogenic effect, pH optimum, temperature range, and stability Saccharogenic 6 amylases produce free sugars upon starch hydrolysis, whereas starch-liquefying amylases breakdown the starch polymer but do not produce free sugars.

Bacillus subtilis Marburg, B. subtilis var. amylosaccharaticus, and B. natto produces saccharogenic α-amylase, where as B. amyloliquefaciens produces liquefying α-amylase. α-Amylases contain a large proportion of tyrosine and tryptophan in enzyme protein and most of them require calcium as a stabilizer. This enzyme is extensively used in different industry (Table 2).

Table 1: Classification of amylases on the basis of glycosidic bond hydrolysis
(Source : Fogarty and Kelly, 1979)

Class	Glycosidic bonds hydrolyzing properties	Examples
1	Hydrolyse α-1,4 bond and bypass α-1,6 linkages	α-Amylase (endoacting amylases)
2	Hydrolyse α-1,4 bond and cannot bypass α-1,6 linkages	β-Amylase (exoacting amylases producing maltose as a major end product)
3	Hydrolyse α-1,4 and α-1,6 linkages	Amyloglucosidase (glucoamylase) and exoacting amylase
4	Hydrolyze only α-1,6 linkages	Pullulanase and other debranching enzymes
5	Hydrolyse preferentially α -1,4 linkages in short chain oligosaccharides produced after hydrolysis of amylose and amylopectin by other amylases	α-Glucosidases
6	Hydrolyse starch to a series of nonreducing cyclic D-glucosyl polymers (cyclodextrins or Sachardinger dextrins)	*Bacillus macerans* amylase (cyclodextrin producing enzyme)

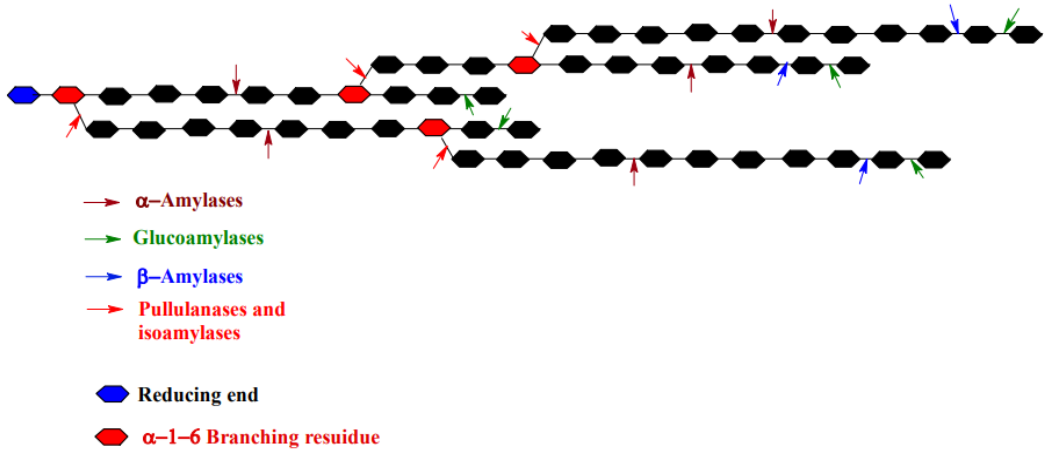


Fig 3: Molecular structure of amylopectin showing specific bonds hydrolysed by various amylases

Table 2: Uses of α-amylase in different industries

Industry	Application
Starch Processing	Liquefaction of starch in the production of sugar syrup
Milling	Modification of α-amylase-deficient strains
Baking	Generation of fermentable sugars in flour, and improvement of crust colour
Brewing	Starch hydrolysis during wort preparation from barley
Paper	Liquefaction of starch without sugar production for sizing of paper
Textile	Continuous desizing at high temperatures
Feed	Treatment of barley for poultry and calf
Biological detergents	Starch removal from food stains
Sugar industry	Breakdown of starch from cane juice to improve filterability

α-Amylase producing bacteria

Bacillus subtilis, B. subtilis var. amylosaccharaticus, B. natto, B. cereus, B. amyloliquefaciens, B. coagulans, B. polymyxa, B. stearothermophilus, B. cladolyticus, B. acidocaldarius, B. subtilis var. amylosaccharticus, B. licheniformis, Lactobacillus, Micrococcus, Pseudomonas, Arthrobacter, Escherichia, Proteus, Thermonospora, and *Serratia* are some α-amylase producing bacteria. However, *Bacillus amyloliquefaciens* and *B. licheniformis* are mainly produced for the industrial production of α-amylase.

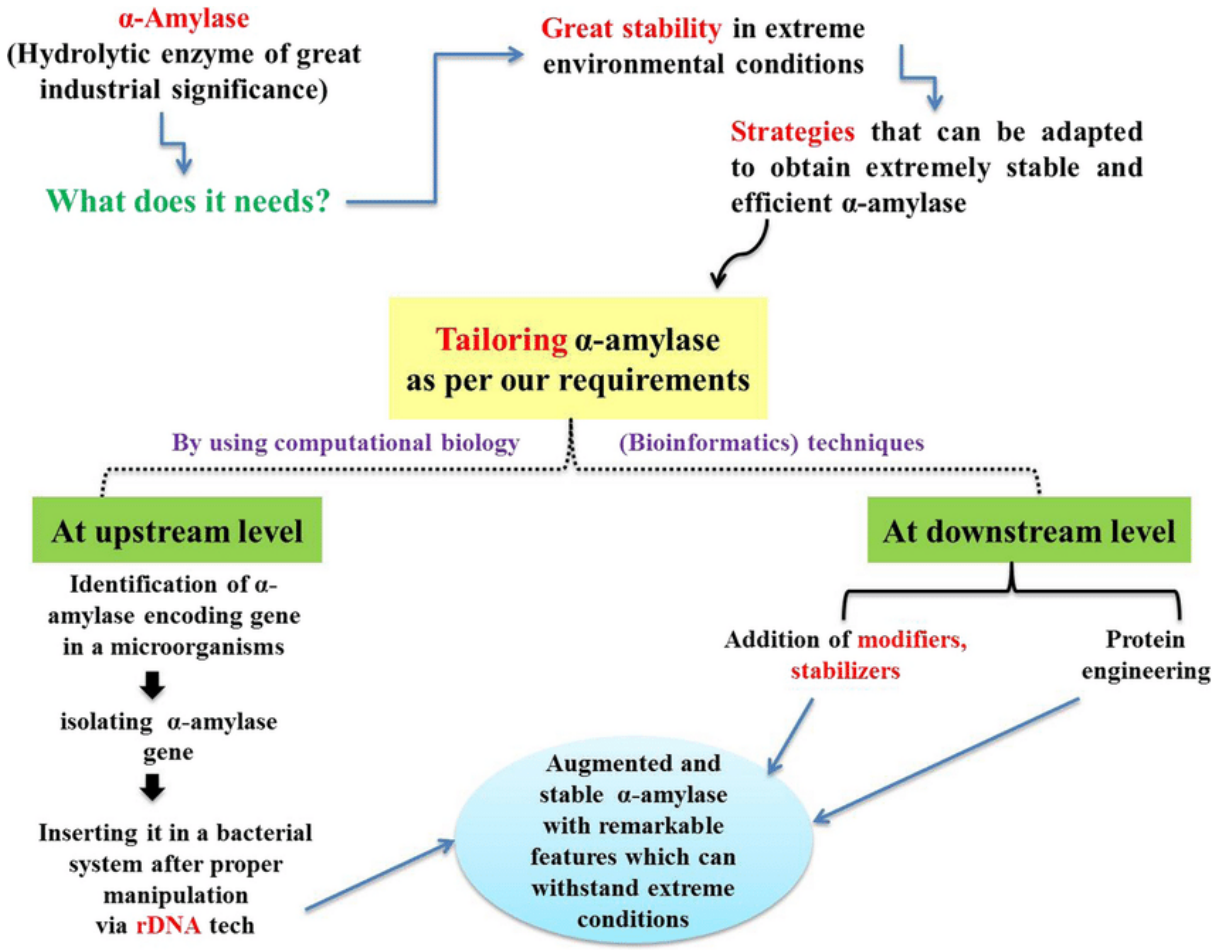
α-Amylase producing fungi

Aspergillus, Penicillium, Cephalosporium, Mucor, Candida, Neurospora and Rhizopus are some α-amylase producing molds and Aspergillus oryzae is one of the mold used as source for the industrial production of α-amylase.

Production of α-Amylase

Bacterial α-amylase

The bacterial α-amylase is inducible and repressible by glucose produced by the hydrolysis of starch. It is assumed that a basal level of the enzyme is produced constitutively, which hydrolyses the starch generating the low molecular weight inducers. The production media for amylase includes 5% starch, 0.5% NH4NO3, 0.28% sodium citrate, 0.13% KH2PO4, 0.05% MgSO4•7H2O, 0.01% CaCl2•2H2O, 0.5% peptone. 0.2% yeast extract and the fermentation is carried out either in batch or in fed-batch manner around pH 6.8. Mostly thermophilic strains are being used for the production of the amylase for applications at elevated temperatures. The temperature of the fermentation process depends on the strain used. In the fermentation process, amylase is produced during the growth of the bacteria and formation of spores.



Fungal α-amylase

Fungi generally produce amylases constitutively but experience repressive effects of some regulators. The fermentation media used for the production of amylase by Aspergillus oryzae contains 8% starch, 1.2% $NaNO_3$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.003% $FeSO_4$, 0.08% $Mg(NO_3)_2$, 0.005% $Mg(H_2PO_4)_2$, and 2.0% malt extract. The fermentation is carried out at 28-30ºC for 3-4 days.

β-Amylases

β-Amylases (α-1,4-glucan-maltohydrolases) are the exoacting enzymes hydrolyzing the α-1,4-glycosidic bonds from the non-reducing ends producing maltose and limit dextrin as the major product and are unable to hydrolyse the α-1,6 branching present in amylopectin. This enzyme is mainly present in plants but some microbes are known to produce this enzyme which includes: *Bacillus polymyxa, B. cereus, B. megatarium, Streptomyces sp., Pseudomonas sp.* and *Rhizopus japonicus*. β-Amylase has been produced on starch waste by a hyper-amylolytic strain of B. megaterium B6 mutant UN12 in Submerged fermentation (SmF) and Solid state fermentation (SSF) (Ray et al. 1997). The starchy wastes from arrowroot, arum, maize, potato, pulse, rice, rice husk, tamarind kernel, cassava, water chestnut, wheat and wheat bran are used as substrate. This enzyme is mainly used in the production of maltose syrup and also digestion of barley starch during beer production.

Glucoamylases

Glucoamylases (α-1,4-glucan-glucohydrolases) hydrolyse starch from the non reducing end producing glucose, maltose, and limit dextrins. Aspergillus niger, A. oryzae, A. awamori, Rhizopus niveus, R. delemar, R. formosaensis, and R. javanicus are the strains employed for the production of glucoamylases. This enzyme is mainly used for the production of fructose syrup and its production is carried out in submerged fermenter. Starch or dextrin induces the production of glucoamylases, therefore, starch is generally added in the production media. The production of the enzyme is carried at 28-30 ºC for 3-5 days depending on the strain. Glucoamylase also catabolite repressible by glucose, glutamic acid and lactose.

1, 6-Glycosidic bond hydrolyzing enzymes

The amylopectin is a branched polymer of glucose chain linked by α-1,6 glycosidic bonds. Pullulanases and isoamylases hydrolyse this bond resulting production of straight chain maltodextrin.

Pullulan is obtained from Pullularia pullulans which is neutral glucan polymer consisting of α-1,4 linked maltotriose unit joined to each other by α-1,6 glycosidic bond. Pullulanases are the enzymes that hydrolyse the α-1,6 glycosidic bond of the pullulan and amylopectins but the isoamylases only hydrolyse the α-1,6 glycosidic bond of amylopectin. Aerobactor aerogens, Bacillus acidopullulyticus, B. polymyxa, Pseudomonas saccharophila, Streptococcus sp., Strptomyces sp., are some of the strains used for pullulanase production. Isoamylases are obtained from Agrobacterium, Erwinia, Staphylococcus, Serratia, Nocardia, Bacillus, Pediococcus, Lactobacillus, and Leuconostoc. Pullulanases and isoamylases are used in the starch hydrolysis process.

Application of amylases

Amylases were the first enzymes to be produced industrially and share 20% of the present enzyme market. Amylases are used in food, feed, textile, and pharmaceutical industries. In the food sector they are mainly used for liquefaction of starch, manufacture of maltose, high fructose containing syrups, oligosaccharide mixture, maltotetraose syrup, and high molecular weight branched dextrins. These products are used for various food preparations (cake, candies, etc) adding characteristic high or low sweetness and maintain texture. The use of amylase has replaced the chemical hydrolysis of the starch as the latter used to yield undesirable by-products and is also uneconomical. Amylase is also used for removal of starch sizer from textile (desizing) making the fabric ready for scouring and dyeing.

Ethanol production from starchy substrates has been improved by using amylases or coculturing the amylolytic strains with ethanol producing microbes in starch based media. This technique has also been used to digest the starch in the brewing process. Amylases are also used for processing waste containing starch generated from food processing plants ultimately reducing the pollution load of effluent and producing microbial biomass protein. Alkaline amylases are used in detergents and dish bar for removing the starch stains on cloths and utensils respectively. Amylases are also ingredient of the digestive syrups used for treating digestive disorders. Amylase treated flour is used for preparing animal feed and have improved digestibility.

8.Suggested Readings

1. Microbiology by M.J. Pelczar Jr., E.C.S. Chan and N.R. Krieg. TMH
2. General Microbiology by R.Y. Stanier, E.A. Adelberg, J.L. Ingram . MacMillan
3. Brock biology of microorganisms by M.T. Madigan, J.M. Martinko, J. Parker. PHI
4. Bacterial metabolism by G. Gottschalk. Springer
5. Microbial physiology by A.G. Moat, J.W. Foster. John Wiley
6. Industrial microbiology by L.E. Cassida
7. Medical microbiology by Greenwood

9. Assignment

1. Two bacteria G=C content same is bacteria same? G=C content advantage and disadvantage of bacterial taxonomy.
2. What is the role of DNA hybridization in bacterial taxonomy? Discuss about the advantage and disadvantage of this methods.
3. What is the role of 16SrDNA in bacterial taxonomy
4. FISH/ PHYLOGENETIC PROBES
5. What are the different methods of bioremediations? Describes the mechanism of resistance of Arsenic and Mercury/ Lead in bacteria
6. What is meant by indicators of bacteria? Describes different methods of studying the portability of water.
7. State the importance of phosphorus in living organisms. Name four genera of microorganisms which can solubilisation process by microorganisms.
8. What is vermicomposting? How is it prepared? Discuss its merits and demerits.
9. Why E. coli. is considered as indicator microorganism for determination of portability of water? How coliform are detected from a water body.
10. What is food poisoning? Discuss at least two food poisoning disease.Brieflydiscuss their possible control.

11. What are the basic chemical types of food spoilage? Briefly discuss various methods of preservation of food.
12. Name two microbes that has important role in finfish spoilage. What are the biochemical changes which occur during finfish spoilage? Discuss various preservation methods of fin-fish.
13. Describe lactic acid/vinegar/lysine fermentation. What do you mean by homofermentative organisms?
14. Describe the recent approaches used to control pollutions associated with toxic wastes from industries.
15. What are microbial plastics and how are these prepared? Distinguish between microbial plastics and synthetic plastics.
16. What are PGPR? Describe the role of PGPR at rhizosphere citing suitable example,
17. What are the role of microbes in bioleaching of copper. Name two bacteria responsible for the process
18. Give an account of green manure. State two advantages and two disadvantage of using green manure.
19. State the characteristic features of the genus Rhizobium spp.
20. What is meant by biogas? How this gas formation is exploited in domestic use?
21. Distinguish between sludge and sewage. Briefly describe any one method of treatment of sludge.
22. Briefly describe the structure and function of nitrogenase gene of Rhizobium sp.
23. Distinguish between bio-remediation and bio-magnification. Give a list of process by means of which different types of pollutants can be removed from the environment.
24. Write a short note on microbial spoilage of milk. What is whey?
25. Discuss various steps of fermentative production of Lysine/ penicillin. Name two antifoaming agents?
26. What is the differences between pasteurization and sterilization? Discuss various types of pasteurization process
27. What is industrial microbiology?
28. Distinguish between industrial and medial microbiology.
29. Name one each antibiotic producing fungal and bacterial species with the name of specific antibiotic
30. What is submerged and continuous fermentation?
31. Mention about the golden era of industrial microbiology

32. Mention the different steps about the downstream and upstream process in fermentation technology
33. Name two type culture collection centre for the source of importance microbes
34. Mention about the role of Louise pasture in development of industrial microbiology
35. Name one each amino acids producing fungal and bacterial species with the name of specific amino acids.
36. Mention about the job duties in industrial microbiology during strain selection
37. Mention deferent techniques followed during strain improvement
38. Define synthetic media and mention its composition
39. What are the different features of microorganism for industrial production
40. Name one sources of enzyme producing industrial microorganism
41. Mention about the lyophilisation techniques for product purification
42. Mention different structural parameters of bioreactor
43. Define synchronous growth
44. Why screening are necessary for the strain selection
45. How do you improve strain for industrial production
46. Mention the role of parasexual cycle help strain improvement

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All the materials are self-written and collected from ebook, journals and websites.