Tulasi Satyanarayana
Bhavdish Narain Johri
Subrata Kumar Das *Editors*

Microbial Diversity in Ecosystem Sustainability and Biotechnological Applications

Volume 1. Microbial Diversity in Normal & Extreme Environments

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Preface

Microbes are the most versatile and adaptable living beings on Earth, which existed for about 3.5 billion years. In the first two billion years, bacteria and archaea dominated the biosphere, colonizing all accessible, normal, and extreme niches. Over their long period of global dominance, bacteria and archaea changed the Earth, transforming its anaerobic atmosphere to one rich in oxygen and generating massive amounts of organic compounds. Eventually, they created an environment that could sustain more complex life forms.

Microbial diversity represents the variability among all types of microbes [prokaryotes (archaea and bacteria), eukaryotes (algae, fungi, protozoa), and acellular viruses and others] in the natural world. Interest in the exploration of microbial diversity has stemmed from the fact that microbes are essential for life because they perform numerous functions essential for the biosphere that include nutrient cycling and environmental detoxification. The vast array of microbial activities and their importance to the biosphere and to human economies provide strong rationale for understanding their diversity, conservation, and exploitation for society.

For long, microbial diversity has been explored by the conventional culture-dependent methods, which allow access of only 0.1–1.0 % of the extant microbes in any of the ecosystems. Over the past two decades, several methods such as rRNA gene sequencing, fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), restriction fragment length polymorphism, and terminal restriction fragment length polymorphism (T-RFLP) have been developed to assess microbial diversity and catalogue microbes without the need for isolation. The use of molecular techniques over the past 20 years has shown that only a very small fraction of microbial diversity so far has been catalogued from all the habitats investigated.

Earth is considered to be inhabited by close to a trillion bacterial and archaeal species and 10–15 million eukaryotic species; this prediction is based on an ecological theory reformulated for large-scale predictions, an expansive dominance scaling law, a richness scaling relationship with empirical and theoretical support, and the largest molecular surveys compiled to date [PNAS (2016) 113: 5970–5975]. The profound magnitude of our prediction for the Earth's microbial diversity emphasizes the need for continued investigation.

Extensive and intensive efforts are being made to understand microbial diversity by both culture-dependent and culture-independent metagenomics approaches.

Despite significant advances made in understanding microbial diversity, most microbes are still only characterized by "molecular fingerprints" and have resisted cultivation. Microbiomic approach is now being adopted for surveying the total microbes present in the different ecosystems (e.g., earth microbiome, ocean microbiome, human microbiome, and rhizosphere microbiome to mention a few). In order to analyze microbial populations in the ecosystems, such as skin and mucosal surfaces of humans and animals, plants, soils, and oceans, new-generation sequencing (NGS) and advanced bioinformatics have become valuable tools. Earth Microbiome Project (EMP) is a landmark study investigating large-scale microbial diversity, which was launched in 2010. Bacterial and archaeal 16S rRNA diversity in 27,751 samples was analyzed from 97 independent studies, which produced 2.2 billion sequence reads [PNAS (2018) 115: 4325–4333]. Two-thirds of EMP reads only could be mapped to existing 16S references that prevented meaningful operational taxonomic unit (OTU) analysis.

By leveraging metagenomics and metabarcoding of global top soil samples (189 sites, 7560 subsamples), it has recently been observed that bacterial genetic diversity is very high in temperate habitats in comparison with fungi and microbial gene composition varies more strongly with environmental variables than with geographic distance [Nature (2018) 560: 233–237]. Fungi and bacteria show a global niche differentiation which is associated with contrasting diversity responses to precipitation and soil pH. Both competition and environmental filtering have been seen to affect the abundance, composition, and encoded gene functions of bacterial and fungal communities, suggesting that the relative contributions of these microorganisms to global nutrient cycling vary spatially [Nature (2018) 560: 233–237].

Good understanding of microbial diversity will allow us to cure diseases, engineer and conserve our environment, manufacture better products, grow more food, colonize other worlds, and much more. In practical and scientific terms, microorganisms give us the power to ask new questions and solve previously intractable problems.

Voluminous data have accumulated on the microbial diversity of various ecosystems. The present attempt is to briefly review the developments in understanding microbial diversity and its role in ecosystem sustainability and biotechnological applications in two volumes of the book titled *Microbial Diversity: Ecosystem Sustainability and Biotechnological Applications* ((1) *Normal and Extreme Environments*, (2) *Soil and Agro-ecosystems*).

In Volume 1, Ramana and his co-workers discussed taxonomy and systematics of bacteria, while Krishna Mohan and his associates described in detail the use of bioinformatics tools in analyzing enormous data related to microbial diversity. Johri et al. dealt with various deterministic factors that shape fungal communities and their probable role in maintaining human, soil, and plant health. Other chapters focus on the diversity of microbes in different ecosystems and their roles in sustainability of different ecosystems and their biotechnological applications.

We wish to thank all the contributors for readily accepting our invitation and submitting well-written chapters in their areas of specialization within the prescribed timelines. The opinions expressed by the contributors are their own. We sincerely hope and wish that the book will serve as a ready reference for the students, scholars, teachers, and scientists in the broad areas of life sciences, microbiology, and biotechnology. Thanks are also due to Springer Nature for publishing the book within a short period for disseminating knowledge from innumerable sources in a volume.

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Dr. B. N. Johri is a former NASI senior scientist at the Department of Biotechnology, Barkatullah University, Bhopal (India). He has received various academic awards, including the Indian National Science Academy's Young Scientist Medal, the Rafi Kidwai Memorial Award and the Katzu Award. He is a fellow of the National Academy of Sciences (I), National Academy of Agricultural Sciences and National Institute of Ecology. He has considerable teaching and research experience, and has 150 research publications and 5 edited books to his credit.

Dr. Subrata K. Das has been a Scientist at the Institute of Life Sciences, Bhubaneswar, part of the Department of Biotechnology, Government of India, since 2003. His main research interests are in microbial genomics and translational research. Dr. Das has discovered 20 novel bacteria, and his metagenomics research has revealed the community composition and functional correlation of microbiome in pristine environment. He has published 54 research papers and contributed a chapter for Bergey's Manual of Systematic Bacteriology. He was selected for the

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Part I

General Aspects

1 Emerging Concepts in Bacterial Taxonomy

Anusha Rai, Indu, N. Smita, G. Deepshikha, K. Gaurav, K. Dhanesh, G. Suresh, Ch. Sasikala, and Ch. V. Ramana

Abstract

Bacterial taxonomy has progressed over the years by virtue of the brisk and competent scientific developments. Ground-breaking molecular techniques have added an edge in the phylogenetic studies, resulting in the quality description of the taxa under studies. New avenues are rapidly developing whose validation has always been embraced and included, which will assist in resolution. It began with the simple application of objective procedures for classification, and now we have arrived at the genome-based taxonomy. This pedantic step has led to the meticulous examination and served to reconcile certain conflicts of the status of the taxa. This field is dynamic and is exploring more options like proteomics and metabolomics in gaining more insights into the lineal heritage. Even though there has been a significant change and addition, there is an ever-growing need for a comprehensive study, which would thread all the attributes together into one functional unit of classification. In this review, we examine the paradigm shift from traditional taxonomy to integrated taxonomy useful in the characterisation of bacteria which in addition aids in the identity of biotechnological targets.

Keywords

Bacterial taxonomy · Polyphasic · Phylogenomics · Integrated taxonomy · Average nucleotide sequence index (ANI)

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1.1 Introduction

The first description of the microorganism in the early 1670s by Robert Hooke and Antonie van Leeuwenhoek under a microscope was the beginning of the field of microbiology. Microbiology then had several dissections and bifurcations owing to novel implications, in various fields such as taxonomy, medicine, agriculture and environment. Like any other field in science, the in-depth and comprehensive knowledge requires the fundamental understanding of the subject under speculation. By implying the term 'fundamental' here, we allude to the application of 'taxonomy' in bacteriology. Bacterial taxonomy at the outset serves as a platform to figure out the basic characteristics of the species and then correlates it with its phylogenetic properties. It deals with the identification of isolates, their classification into the taxa and creating new ones (if novel) and their nomenclature which are carried out in accordance with the rules and regulations laid down in the Bacteriological Code 1990 (revised in 2008 by Parker et al. 2018). Classification and identification has been served best with polyphasic studies, whereas the nomenclature has always been advised to imply and reflect the genomic association. It is as essential as any other discipline of the biological sciences because it provides a scientific framework for the salient understanding of the bacterial species.

Taxonomy and systematics have been often used interchangeably, but there lies a thin line that governs a difference between the two. Taxonomy is based on practical classification dictated by theory, whereas systematics is the evolutionary study of the diverse group of organisms and its related taxa. Conventionally, bacterial taxonomy helps us to picturise the evolutionary history and its concordant relationship with the nearby organisms. Bacterial taxonomy is aimed at achieving authentic, reliable and reproducible knowledge ready for dissemination. As of today, the newness of the bacterial taxa is determined by the phylogenetic status of an isolate mostly using the 16S ribosomal RNA (rRNA) gene in supplementation of the phenotypic and chemotaxonomic properties of the culture. Although conscious efforts are meticulously made to describe and define taxa validly, still there are gaps which need to be filled with the help of upcoming techniques. The present system of bacterial taxonomy has progressed and developed due to inclusions in the light of various taxonomical methods. It has also led to the unearthing of new valuable taxa thus giving microbiology a new dimension.

1.2 Historical Developments

Bacterial taxonomy became the most sought-after subject in the field of microbiology after its inception in the early 1600s due to quantum surge in the discoveries and inventions, thus escalating the accumulation of knowledge. Understanding bacteria at the surface level became futile which genuinely leads to the importance in the definition of the taxa under study. This ultimately led to the development in the robust methods for the taxonomy in bacteriology. Even without the advent of molecular work, various attempts were made consciously for the classification

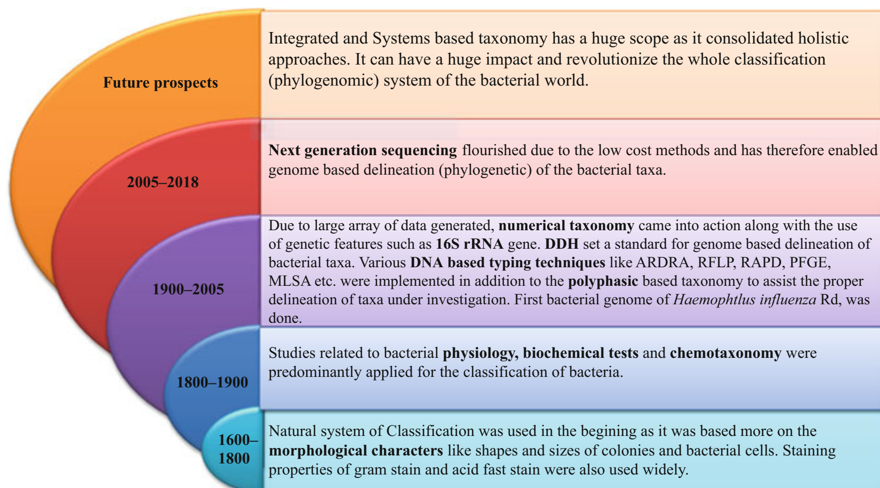

Fig 1.1 Diagrammatic representation of evolution of the classification systems extensively used in different era for appropriate delineation of bacterial taxa

although it was discreetly based on morphology, therefore always trying to systematise and correlate its phenetic with phylogenetic characteristics and its significance. Partitioning domain bacteria into various taxa levels proved beneficial and productive. Till date, various methods have been incorporated and improvised for the enhancement of the bacterial taxonomy. Direct or indirect contributions by scientists throughout the history of biological sciences made it possible, and therefore, we see that one event led to another, thereby causing the definitive birth of bacterial taxonomy (Fig. 1.1).

The development of bacterial taxonomy can be traced into different phases:

Phase I (1600–1900 AD)

Taxonomic study during this phase was based on simple biological observations and morphological descriptions. It was in this era that Antonie van Leeuwenhoek and Robert Hooke first observed ‘animalcules’ like structure under the single-lens microscope in the early 1670s. In the next decade of the 1800s, maximum contributions were made to aid the morphological studies. Muller and his contemporaries played a major role in promoting taxonomy as they inherently perceived the importance of assigning taxa genuinely owing to its towering application. Koch developed agar plate technique for the isolation of pure cultures, making the isolation of bacterial species convenient. In 1872, Ferdinand Cohn proposed that bacteria could also be designated as genus and species. Assorted methods like acid-fast stain and Gram staining were developed for understanding the morphology by Paul Ehrlich and Christian Gram, respectively. Further, for the adept usage, petri plates were developed by R. J. Petri. These scientific landmarks improved the

morphological studies and paved the ways for the future development in molecular-based taxonomy.

Phase II (1900–1980)

This phase saw the emergence of the application of biochemical and physiological properties for the taxa descriptions in accordance with the report presented by the Society of American Microbiologists (later changed into American Society for Microbiology) in 1923. This report also served as the ground for the publication of the first edition of *Bergey's Manual of Determinative Bacteriology* by David Hendricks Bergey. An exclusive journal was then established at the fifth International Congress for Microbiology in 1950 as the *International Bulletin of Bacteriological Nomenclature and Taxonomy* (IBBNT) and then later renamed the *International Journal of Systematic Bacteriology* (IJSB) in 1966. It was only in 2000, that it was called *International Journal of Systematic and Evolutionary Microbiology* solely for the description of valid taxa (Oren 2015). Numerical taxonomy was widely used owing to the large datasets arising from the characterisation. The coming years saw the emergence of semantides (or semantophoretic molecules which are biological macromolecules that carry phylogenetic information about evolutionary history) and its applications. DNA-DNA hybridisation was widely applied from the 1960s by various groups and is still considered as the golden standard for the species description. Polyphasic term was first coined by Colwell (1970), where the combined approach of phenotypic, genotypic and chemotypic characterisation was applied for the genus *Vibrio*. It was finally in 1977 that Carl Woese implied the use of ribosomal RNA (16S rRNA) sequence to identify *Archaea* and *Bacteria*.

Phase III (From 1980 Till Now)

This phase saw the dawn of the genomic era and its wide application in the demarcation of the taxa. The following years showed marked development in the genomic techniques for the better understanding of the genome content. Walter Gilbert and Frederick Sanger in 1977 made the sequencing less tedious by initiating methods for DNA amplification (Heather and Chain 2016). Various DNA typing methods were applied for the determination of inter- and intraspecies relatedness (Stackebrandt et al. 2002). The years after 1980s saw the emergence of techniques targeting fractions of whole genome. Randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), pulse-field gel electrophoresis (PFGE), ribotyping and amplified ribosomal DNA (rDNA) restriction analysis (AFLP) were widely celebrated and employed for delineating bacterial taxa. Further in 1998, multilocus sequence typing approaches (Maiden et al. 1998) and coupled in silico bar coding (Shivali et al. 2012) were used for annotation of genomic relatedness.

It was in the year 1995 that the first bacterial genome of *Haemophilus influenzae* was sequenced by Fleischmann's group (Fleischmann et al. 1995). This was a breakthrough in the genomic era owing to significant developments contributed by

the molecular biologists. Thus it began the genomic era resulting in the higher resolution in the genome analysis. Focussed genome studies are now possible owing to the marked development in molecular techniques along with advancement in the bioinformatics tools. Recently, a digital protologue database was designed in order to make a reposition of all the newly described species or the emended taxa (Rosselló-Móra et al. 2017). According to the EzBioCloud database, 63,587 16S rRNA gene sequences have been deposited, and 92,802 genomes have been sequenced. Out of which, 23.04% (14650) are valid names, 0.81% (515) are invalid names, 0.46% (292) are *Candidatus* taxa, and the rest of 75.69% (48129) are the phylotypes of the total bacterial taxa (www.ezbiocloud.net/).

1.3 Importance of Bacterial Taxonomy in Applied Sciences

Taxonomy aids scientific communication as it allows the scientists to make predictions and frame hypotheses about the organisms. Often microbiologists use informal names like purple bacteria, sulphur bacteria and spirochetes, for example. However, in the scientific classification, each organism/species is assigned to a genus using a two-part binary name written in italic (underlined when handwritten) with a majuscule first letter with the exception of sobriquets for species and subspecies, e.g. *Escherichia coli*. The most widely accepted prokaryotic classification by microbiologist's community appeared in the early 1990s in the *Bergey's Manual of Systematic Bacteriology* as 'Taxonomic outline of the Prokaryotes' aiming to aid in the identification of species. For bacterial taxonomy, valid name must be in Latin or Neo-Latin using basic Latin letters only. Many species are named after person, either discoverer or a famous person in the field, for example, *Shivajiella* is named after Dr. Shivaji, an eminent Indian microbiologist who has made a significant contribution to our knowledge of heterotrophic bacteria from different predominantly cold habitats worldwide (Kumar et al. 2012). Many species (the specific epithet) are named after the place they are present or found, for example, the specific epithet of *Rhodomicrobium udaipurense* is named after the place (Udaipur) from where it was isolated (Ramana et al. 2013).

Usefulness of bacterial taxonomy to its core is evident by the heterogeneity in the metabolism of strain variation. For instance, from an evolutionary point of view, the species of the genus *Shigella (S. dysenteriae*, *S. flexneri*, *S. boydii, S. sonnei)* are strains of *Escherichia coli* (polyphyletic), but due to the difference in the genetic make-up of the pathogenic strains, they cause different medical conditions. *Escherichia coli* is a poorly classified species since some strains share only 20% of their genome. Being so diverse, it should be given a higher taxonomic rank. But, due to the maladies associated with the species and to avoid confusion in medical context, it remained unchanged. According to the National Centre for Biotechnology Information (NCBI), 3180 strains of *Escherichia coli* were reported (of which 2383 strains have their genome sequenced). Merely calling *E. coli* will not assure the organism under consideration. Thus, identity of the organism to its core (up to the

strain level) is crucial before the organism is taken up either to industry or for research purpose.

There are cases where investigators misidentified the species resulting in taxonomic errors in classification. For instance, genus *Agrobacterium* is nested under *Rhizobium* based on molecular data. Thus, *Agrobacterium* species are transferred to the *Rhizobium* genus resulting in *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens)*, *Rhizobium rhizogenes*, *Rhizobium rubi*, *Rhizobium undicola* and *Rhizobium vitis*. But, due to the plant pathogenic nature of *Agrobacterium* spp., maintaining the genus *Agrobacterium* was proposed and later counterargued. Similarly, in the order *Pseudomonadales* (*Gammaproteobacteria*), the genera *Azotobacter* and *Azomonas macrocytogenes* (true members of the genus *Pseudomonas*) were misclassified due to nitrogen-fixing capabilities and the large size of the genus *Pseudomonas* thus rendering classification problematic. Also, the *Bacillus* species of the phylum *Firmicutes*, belonging to the '*Bacillus cereus* group' (*Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus cereus* and *Bacillus medusa*), have 99–100% 16S rRNA gene sequence similarity (97% being commonly cited acceptable species cut-off) and are polyphyletic but for medical reasons retained separate. Also, there are cases where investigators rectified the errors in taxonomic classification. For instance, *Deinococcus radiodurans* was originally classified as *Micrococcus radiodurans* by Anderson et al. (1956) due to its similarity with the genus *Micrococcus*, but later on, it was renamed *Deinococcus radiodurans* based on polyphasic data (Brooks and Murray 1981). Therefore, we see that precise bacterial identification is crucial for taking an organism for any study and thus avoiding any taxonomic error.

1.4 Prevailing Methods for Classification

Bacterial taxonomy first started with a vision of resolving its physical affiliation to its phylogeny in order to correlate its genomic imprints. The phenotype-based taxonomy led to the enormous addition of bacterial species because only few morphological factors were considered for the classification. More than 90% of all the species described in *Bergey's Manual* were subsequently reduced, and only species included on the approved lists of bacterial names became validly named species (Skerman et al. 1980; Garrity 2016). Major amendments occurred due to the use of DNA-DNA hybridisation (DDH) and 16S rRNA gene applications. Hence, transitioning from simple to holistic approaches, bacterial taxonomy has come a long way. It has still left a room for numerous improvisations owing to the gradual advancement in science.

Till date, polyphasic approach has been relevantly applied for the taxonomic purpose. It includes chemotaxonomic features (cell wall components, quinones, polar lipids, etc.), morphology, staining behaviour, culture characteristics (medium, temperature, incubation time, etc.) and genetic properties (G + C content, DDH value, 16S rRNA gene sequence identity with other closely related species) (Tindal et al. 2010). In some cases, DDH values have been strictly advised to strengthen taxa

delineation. According to the report by the Ad Hoc Committee of the International Committee for Systematic Bacteriology issued in 1897, the following parameters could be used for valid taxa description: (a) phenotypic (b) chemotaxonomic and (c) genotypic properties.

(a) *Phenotypic*

Phenotypic methods form the basis for formal description of taxa, from species and subspecies up to genus and family level (Garrity 2016). Traditional phenotypic tests used in classical microbiological laboratories include characteristics of organism on different growth substrates and growth range in different conditions such as pH, temperature, salinity and susceptibility towards different antibiotic stress (Prakash et al. 2007). Phenotypic data which are analysed by using computer-assisted numerical comparison is known as numerical taxonomy. Phenotypic data matrices showing the degree of similarity between each pair of strains and cluster analysis resulting in dendrogram revealed a general picture of the phenotypic consistency of a particular group of strains. The advantage of phenotypic characterisation is that they can be easily observed, scored and measured without using any expensive technology. As the phenotypic characteristics depend on the conditional nature of gene expression, the same organism may show different phenotypic characteristics under various environmental conditions. Therefore, phenotypic data must be compared with a similar set of data from type strain of closely related organisms (Tindal et al. 2010).

(b) *Chemotaxonomy*

The term 'chemotaxonomy' refers to the application of analytical methods based on various chemical constituents of the cell to classify bacteria (Komagata and Suzuki 1987; Tindal et al. 2010; Sutcliffe 2015). It has enabled the establishment of specific chemical markers for proper classification and identification. The most commonly used chemical markers include cell wall/membrane component such as peptidoglycan, teichoic acid, polar lipid composition, relative ratios of fatty acid, sugars, lipopolysaccharide, isoprenoid quinones, carotenoids, chlorophyll composition, polyamines and fermentation products. Peptidoglycan can be an excellent marker for the taxonomic studies as it is present in most of the phyla, even including *Planctomycetes* and *Chlamydiae* according to recent studies (Pilhofer et al. 2013; Liechti et al. 2014) except for *Mycoplasma* (Rottem and Naot 1998).

Bacteria vary in their membrane lipid composition; therefore, polar lipids are considered for classification and identification of bacteria. Various chemical structures of fatty acids have been identified. The variability in chain length, double-bond position and substituent groups has proven to be very useful for the characterisation of bacterial taxa. Fatty acids are also the major constituents of lipids and lipopolysaccharides in microbial cells and have been therefore extensively used for taxonomic purposes. The process is termed the fatty acid methyl ester (FAME) analysis. Often fatty acids of variable length between 9 and 20 are considered for

classification (Sharmili and Ramasamy 2016). Hopanoids are pentacyclic triterpenoid sterol-like membrane lipids (Belin et al. 2018). Since hopanoids preserve source-specific information and can be linked with specific taxonomic group, physiological process, metabolic process or environmental condition, they can be used as a lipid biomarker (Cvejic et al. 2000; Blumenberg et al. 2012; Silipo et al. 2014). Hopanoids as chemotaxonomic markers were used in some of the recent studies (Tushar et al. 2015).

Isoprenoid quinones are components of cytoplasmic membrane of bacteria. Due to inconsistency of isoprenoid quinones along with difference in hydrogenation, saturation and side chain length, it acts as signature molecule for characterisation of bacteria at different taxonomic levels (Nowicka and Kruk 2010). Distribution of polyamines is universal in bacteria with significant quantitative and qualitative difference due to which they can be used as suitable chemotaxonomic markers. Depending on the group of organisms studied, polyamine patterning is being used to trace relatedness at and above the genus level and at the species level.

Whole-cell protein pattern can be analysed and compared for grouping of many closely related strains. Numerous studies have revealed a correlation between high similarity in whole-cell protein content and DNA-DNA hybridisation (Jarman et al. 2000). Identification is based on the comparison of the spectral database containing peptide mass fingerprints with the type strains by using the technique of MALDI-TOF (matrix assisted laser desorption/ionisation-time of flight) [Singhal et al. 2015]. It is applied to diagnose commensal bacterial species of *Enterococcus* sp. and *Escherichia* sp. by the determination of their unique spectra (Santos et al. 2015). Fourier transform infrared (FTIR) spectroscopy, on the other hand, uses the inherent property of the organism to produce specific metabolites to identify at the species and strain level (Naumann et al. 1991). When the whole microbial cells are excited by the absorption of the IR radiation, then it produces vibrational properties specific to the chemical bonds produced (Carlos et al. 2011). Metabolomic techniques like FTIR are often being used for the quick identification of bacteria on the basis of their particular metabolic fingerprints (Venkata Ramana et al. 2013). Biolog MicroPlates exploit the bacterial metabolism process for the utilisation of carbon sources. Species may be identified by specific colour change on the plate based on the metabolic fingerprint (Vehkala et al. 2015; Al-Dhabaan and Bakhali 2017). Lipidomes (Srinivas et al. 2016) and fermentomes (Sravanthi et al. 2016) are some of the chemomics used in recent bacterial taxonomy.

(c) *Genotypic*

The genotype-based methods have completely changed the scenario in the bacterial systematics world. It has finally assisted to draw lines between the various taxa levels. It mostly focusses on the retrieving of genomic information like DNA-DNA hybridisation, G + C contents, rRNA gene sequence analysis and DNA-based typing methods (DNA fingerprinting). DDH is required when a new taxon shares more than 97% 16S rRNA gene sequence similarity (Tindall et al. 2010). Value equal to or higher than 70% has been recommended for the definition of members of a species

(Wayne et al. 1987). The GC content is the calculated percentage of GC in the genome and therefore varies from one organism to another. Within prokaryotes, the G + C content varies between 20% and 80%. If the phylogenetic studies of an isolated strain reveal approximately 6% 16S rRNA gene sequence difference with its other closely related genus, then it can be recommended to represent the novel genus (Yarza et al. 2008).

It was considered that bacterial strains can be delineated with the data on 16S rRNA gene sequence analysis wherein the strains that show more than 3% sequence divergence are considered to represent different species (Rosselló-Móra and Amann 2001). However, with good quality and near full-length sequences, the value has been revised to 98.7–99% (Sackebrandt and Ebers 2006). A bacterial species can be properly defined as the group of strains sharing 70% or more DNA-DNA hybridisation with 5 °C or less ΔT_m value (T_m is the melting temperatures of the hybrid) among members of the group (Grimont 1981; Wayne et al. 1987). DDH is deemed necessary when strains share >98.7% 16S rRNA gene sequence identity. However, DDH has its own disadvantage because of which it cannot be applied to all the genera of prokaryotes. The difference in the sequences must be strongly supported by its distinctive characteristics. When a genetically close organism diverges in phenetic characteristics, then it can be ranked as a subspecies (Wayne et al. 1987).

The ribosomal locus such as the internal transcribed spacer region which is located between the 16S and the 23S rRNA genes has been scrutinised for the phylogenetic properties. Although this technique can outline the species/strain level but still at the lower level, it remains incongruous (Valera and Garcia-Martinez 2000). Multilocus sequence typing has been mostly used in epidemiology and pathological purposes but still generated its place in bacterial systematics. It has out sided the traditional procedure for determination of the genomic relatedness at inter- and intraspecific levels by sequence profiling of housekeeping genes (Maiden et al. 1998). The advantage of it not only lies in the application of the cultivable species, but also to those which are difficult to cultivate (Martens et al. 2008). Here, 6–11 housekeeping genes of the microbial species are profiled, which are around 470 bp long and stably selected. Amplified ribosomal DNA (rDNA) restriction analysis (ARDRA) can be used for the characterisation of bacterial isolates and has potential for analysing mixed bacteria communities. It is based on the principle of conserved restriction sites on the 16S rRNA which forms particular phylogenetic patterns specific to certain taxa (Abed 2008). The obtained banding pattern serves as a fingerprint for identification of respective bacteria. BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR) techniques play a vital role in the studies of microbial isolates from various environments.

The sequencing of the *Haemophilus influenzae* genome was a landmark in modern biology, as it marked the beginning of the genomic era. Next-generation sequencing (NGS) technologies introduced from 2005 provided a new platform resulting in a rapid increase in the prokaryotic genomes getting sequenced (Deurenberg et al. 2017; Besser et al. 2018). Genomic taxonomy is the newest

addition to the bacterial systematics world. Genome microbial taxonomy is paving a new path for the dynamic system-based classification.

1.5 Advanced Genome-Based Bacterial Taxonomy

Polyphasic taxonomy complemented along with the molecular fingerprinting techniques (AFLP, RFLP and others) served for the delineation of the taxa for a long time (Rademaker et al. 2000; Gurtler and Mayall 2001; Van Belkum et al. 2001). There are certain gaps generated in the definition of species which remain to be duly filled. Genome-based taxonomy is the missing link and can bridge the gap between the genome and phenotype-based classification. It has been well proven that genomic signatures can be tapped for the definition of bacterial species. A uniform definition of the bacterial species on the establishment of genomics would be to consider the strains from the same species.

With all the advantages that each technique has added in this field, the whole-genome sequencing will be the ultimate step for the resolution of taxonomic position of prokaryotes. The fine advancement in technology and the reduction in the cost of the whole-genome sequencing have led to the monumental shift in the sequencing of the genomes. There are thousands of whole-genome sequences of prokaryotes available, but still only a few hundred are of the type strains, therefore greatly restricting the use of genomic data for the comparative use in taxonomy (Chun and Rainey 2014). Therefore, the governing body has made mandatory in some cases for the whole-genomic sequencing where it would be used to break the incertitude situation in outlining its taxonomic rank (Konstantinidis and Tiedje 2005a). Although not mandatory for publication, the inclusion of this genome sequence data is highly recommended and will be expected to include new taxa descriptions submitted to *International Journal of Systematic and Evolutionary Microbiology*.

In the recent years, whole-genome sequencing has assisted in solving the complex taxonomical positions of certain species of *Vibrio*, *Mycoplasma*, *Xanthomonas* and *Prochlorococcus* (Jones et al. 2004; Thompson et al. 2013; Barak et al.2016). On the basis of genomic parameters like average amino acid sequence identity (AAI) and average nucleotide sequence identity (ANI), data has been applied for the bacterial species definition and classification (Qin et al. 2014). The ANI of common genes between strains being compared is especially closely correlated with the level of DDH, and a 95–96% ANI value can serve as a genomic measure for prokaryotic species delineation (Konstantinidis and Tiedje 2005b). At the genus level, the percentage of conserved proteins (POCPs) was used for the robust indexing of the genus boundary for the prokaryotic group. If all the pairwise POCP values are higher than 50%, then it could be defined as prokaryotic genus (Qin et al. 2014).

Vibrios belonging to the *Gammaproteobacteria* are found in the surrounding environment and also in close association as pathogen with the plant and animal. There are around 152 species of the genus, most of which are in specific host-pathogenic relationship whether it be human or animals and some in mutualistic

relationship (http://www.bacterio.cict.fr/index.html). Often techniques like MLSA, DDH and ΔT_m are often conventionally used for the delineation of the *Vibrio* species (Thompson et al. 2004). They are usually difficult to segregate into taxa owing to similar genomic and phenotypic characteristics. In a recent study, Thompson et al. (2009) restructured the *Vibrio* genus by a study comprising 43 genomes and observed that vibrios were distributed into three major groups or genera of *Vibrio*, *Photobacterium* and *Aliivibrio*. Critical genome analysis of vibrios has evidently revealed the description of two novel species which were closely related to *Vibrio cholerae*. Similarly, species closely related to *Mycoplasma* are very difficult to delineate on the basis of their 16S rRNA similarity and DDH. For example, *Mycoplasma pneumoniae* and *Mycoplasma genitalium* have a 16S rRNA similarity of about 98%. When critical analyses of 46 different genomes of Mycoplasmas were done, it was observed that *Mycoplasma pneumonia* and *Mycoplasma genitalium* had only 73% MLSA similarity, 67% AAI and 88 Karlin genomic signatures. With many more observations based on genome, *Mycoplasma* was seen to be paraphyletic (Thompson et al. 2013).

More evidences need to be generated using core genome for structuring the flexible positions of few species. Another interesting case is that of *Mycobacterium*. Till date, there are 193 species and 13 subspecies validly described (http://www.bacterio.net). *Mycobacteriaceae* consist of pathogenic as well as non-pathogenic species. *Mycobacterium tuberculosis* and *Mycobacterium leprae* and *Mycobacterium abscessus* are considered as pathogenic, whereas *Mycobacterium smegmatis* and *Mycobacterium thermoresistibile* are non-pathogenic (Brosch et al. 2000; Prasanna and Mehra 2013). Miscellaneous software for bioinformatics especially designed for the analysis of genomes makes the annotation of several mycobacterial species feasible. In the present scenario, the advancement has led to the collective information in relation to the evolutionary traits, sequence homology, conserved regions and gene ontology content (Malhotra et al. 2017). The study of comparative genomic analysis of 21 mycobacteria conducted by Zakham et al. (2012) revealed that 1250 *Mycobacterium* gene families were conserved across all species. The *Mycobacterium* pan-genome showed a total of 20,000 gene families (Zakham et al. 2012). Moreover, it was seen that the pathogenic ones had undergone genome reduction and gained defined group of genes for repair and protection (Wassenaa et al. 2009; Zakham et al. 2012). *Mycobacterium leprae* is the pathogenic one with the diminutive genome with 1600 genes and approximately 1300 pseudogenes (Singh and Cole 2011). Functional orthologs of these pseudogenes (>75%) were present in other mycobacterial species belonging to various protein groups (Malhotra et al. 2017; Muro et al. 2011). Therefore, tapping these variations for the genomic identity can be an excellent tool for the taxonomic purposes.

Coleman and Spain (2003) first described *Mycobacterium* strain JS623 from the environmental sample based on the identity value of 96.7% (421 bp) 16S rRNA being more similar to *Mycobacterium smegmatis*. It was still studied as the strain under the *Mycobacterium smegmatis* species until Ramasamy et al. (2014) increased the species limit of delimitation to 98.7%. Subsequently undertaken methodical gene and genome analyses showed that strain JS623 is a mycobacterium more related to

M. moriokaense than to *M. smegmatis* and indicate that it is not a member of this last species, as was previously believed (Garcia and Gola 2016). Therefore, JS623 was probably not a member of *M. smegmatis*.

We see that standard methods like 16S rRNA gene sequence analysis and DDH might not be superior in terms of establishing phylogenetic relationships. It has to be corroborated with other references generated from whole-genome sequencing. Genome-based taxonomy becomes very essential for the delineation of the closely related species in order to disclose the species-specific patterns. In terms of genomics, a collate prokaryotic species can be defined as the strains from the same species which share <10 in Karlin signatures (Karlin et al. 1997; Coenye and Vandamme 2004), > 95% AAI and ANI (Goris et al. 2007; Konstantinidis and Tiedje 2005a, b; Rohwer and Edwards 2002), > 95% identity based on multiple alignment genes (Thompson et al. 2008) and > 70% in silico genome-to-genome distance (Auch et al. 2010).

1.6 Future Prospectives

We have seen the expansion of bacterial taxonomy, mainly due to the contributions of more of accessible and unambiguous techniques. Further advent of molecular techniques added to their improved taxonomic studies. Now, we are in the transitional stages of omics wherein a lot of data generated shall be subdivided and specific markers shall be applied for the taxa studies. Already, huge genome data have been submitted in various databases. The Genomic Encyclopaedia of Bacteria and Archaea (GEBA) project is started by the DOE Joint Genome Institute in 2007 to pilot genome sequences of the type strains (Wu et al. 2009). This effort will definitely bridge in the gap which has arisen from the biased sequencing of only the physiologically advantageous ones. It has been suggested that time and again that the type strains of genomes can be used for comparative studies for taxonomical purposes (Chun and Rainey 2014). Whitman also emphasised on the future complementation of DNA and genome sequences to some extent to substitute the pure cultures as type materials to be deposited in culture collections (Whitman 2015). Moreover, the genomes reflect the biology of the organism and its evolutionary lineage pattern, therefore avoiding fabrication of redundant species. The expansion of the next-generation sequencing and its subsequent decrease in the price have resulted in the better understanding and scanning of gene families for fine delineation of the taxa. Dynamic approaches are being embraced for the accelerated taxonomical purposes of bacteria. Innovative technologies and study systems are underway for effective identification. The following systems are highly ambitious and visionary, thus enabling the filling of gaps and errors in the taxonomic studies. Advanced taxonomy can be summarised as follows:

(i) *Integrated Taxonomy*: Although polyphasic taxonomy has solved the problem of ambiguous ranking of the taxa by its methodical application of phylogeny, chemotaxonomy and phenotype-based studies, there remains a generous gap

for upgradation. Polyphasic studies have added some dimension to taxonomy, but it has remained confined to certain aspects as it does not reflect the genomic content of the organism. Incorporation of various metabolomic and physiological affixes in polyphasic taxonomy has its own impediment owing to the variability caused by the environmental differences. All the more, the attributes considered for classification remain boxed and not interlinked inherently with the genomic content. Therefore, there is a certain need for an immediate classification system that would encompass genome data for the formulation of new taxa. The unparallel system for such kind of taxonomy would be that of 'integrated taxonomy'.

In the present context, scientists are exploring the aspects of genomics, transcriptomics and metabolomics to elucidate various processes and functions. Whole-genome sequencing plays a vital role in describing bacterial phylogeny through systems biology approach by their mechanistic genome annotation. Application of the same genomics for the translational-based studies would lead to the legitimate discerning of the relative species taxa based on the data generated. This type of integrated studies will help us assemble diverse information and put forth re-analysed phylogenetic history and novel biological proteins (Wu et al. 2009). Genome annotation of the taxa under study can ravel huge information owing to large generation of dataset. Translational and prediction-based inspection of the genome sequences can help us ascertain the production of possible novel metabolites and proteins specific to the taxa. Thus, all these compendious works shall complement 16S rRNA gene base for describing bacterial phylogeny with added values.

In the next-generation bacterial identification (NGBI), taxonomist would rely on both genomics and metabolomics for determining the microbial phylogeny. Postgenomic developments are, therefore useful in describing the phylogeny in a more determined way. Taxonomy, especially the bacterial one, has lawfully accepted the new advancing technologies. Therefore, polyphasic taxonomy can be clearly replaced by the integrated taxonomy.

(ii) *Systems Taxonomy*: Even with the integrated taxonomy providing the genome framework for classification, yet system-based taxonomy remains the advisable and prudent system for classification. It is an ultimate and ambitious goal to have inclusive taxonomic studies embracing genomics, proteomics and metabolomics along with various other components. It would in fact consider all the factors which influence the survival of the bacteria under study. It is not too far owing to the paramount advancement in the technologies for the development of system-based taxonomy wherein compendious interdisciplinary subjects are included for the comprehensive yet precise functional taxonomy.

Generally, it would be a holistic approach to identify and assign taxa to the bacteria under studies. Microbial metabolomics, proteomics and transcriptomics are variable and dynamic in nature under different environmental conditions, but still they play a vital role in bacterial physiological processes. So far, all these factors are

not considered for taxa delineation because of which we are failing to understand certain evolutionary divergences and convergences leading to speciation. System-based taxonomy can be the missing link for critical studies for taxa delineation. Moreover, systems-based taxonomy constitutes all the consolidated component of systems biology that affects the organism's existence. It would as such consider all the parameters encompassing the taxa citing from a single cell to that of its complex interaction in the environments. Starting with the cell architecture (shape and size), its membrane components, biochemical activities, metataxon information (polar lipids, quinones, etc.) and physiological processes (growth mode, respiration, reproduction and energy metabolism) to its genomic fingerprints all would be considered. At another higher level, all the proteomic and metabolomic networking along with its ecological habitats and environmental factors (such as pH, temperature and salinity) shall be connected. Basically, these types of studies would network all the possible factors together into functional units for the proper understanding of the taxa under study and subsequently describe them into novel ones. Therefore, we see that this type of system-based taxonomy can solve major issues arising out of biased studies.

(iii) *Virtual Taxa*: It is evident from numerous studies that less than 1% of the actual microbial wealth is known, whereas the remaining part lies undiscovered. Metagenomics has also strengthened the same scientific belief of yet to be uncultivated microbial wealth. Therefore, forming ranks with the help of virtual taxa would be the most appropriate need of the hour. Particularly, it would further strengthen the taxonomy of the uncultured or the *Candidatus* species status; therefore, virtual taxa can be well correlated. It is also at par with the *Candidatus* species status as it is strenuous to cultivate them. Virtual taxonomy can be defined as the identification and classification of single-cell bacteria based on its genomic DNA and other cellular parameters under consideration. Single cell, screened using fluorescence-activated cell sorting (FACS), can be genome sequenced to frame the virtual taxonomy of the organism using bioinformatics tools. This tendency can be targeted to make use of *Candidatus* species for studying the community analysis.

As such genome sequences would serve as the main source of information in postulating and circumscribing species that are not available as pure cultures (Konstantinidis and Rosselló-Móra 2015). Based on the DNA sequences retrieved from the metagenomic studies, virtual taxonomy could be functional and practical in which prediction and conclusive studies based on the genome information about its cell structure, physiology and biological roles could be reported (Fig. 1.2). Combining and analysing the results thus obtained will fetch 'consensus taxonomy' of a microorganism. As a consequence, there is an immediate need of non-culture-based rapid method of identification utilising molecular approaches mainly centring DNA-based methods selecting taxa-specific loci. This propensity will foster a rapid and cost-effective method for bacterial identification in the coming years.

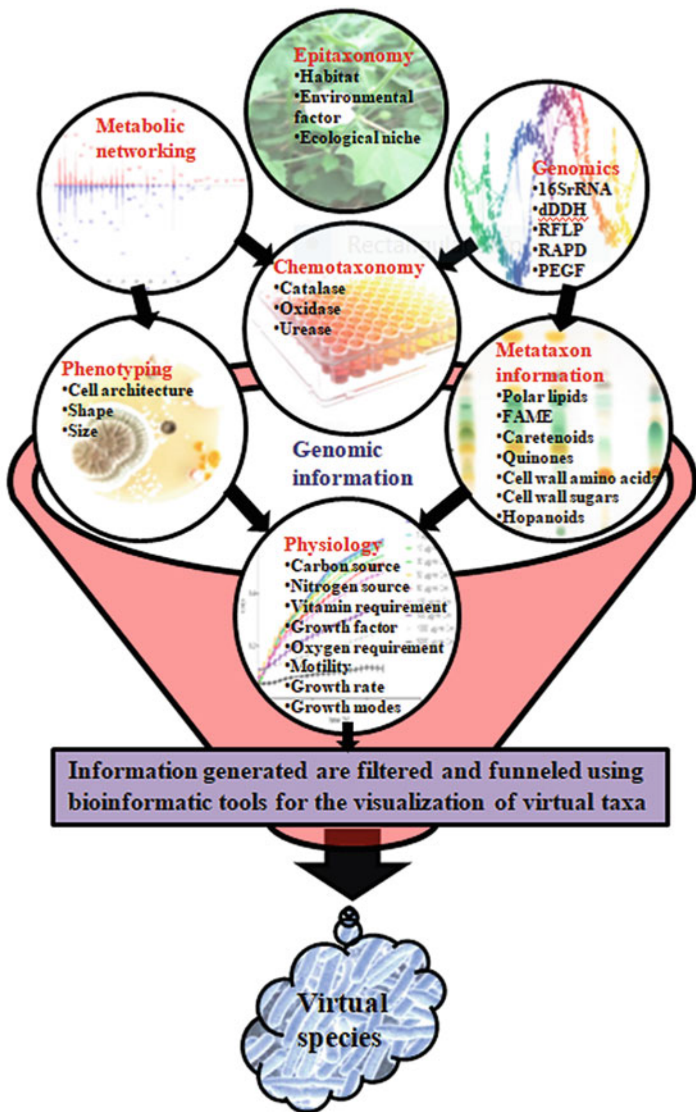

Fig. 1.2 Identification and classification of finely delineated virtual species by filtration of the epitaxonomic information and genomic information such as genomics, phenotyping, chemataxonomy, metataxon information, physiology and metabolic networking, using bioinformatic tools

1.7 Concluding Remarks

Since all the information applied in the taxonomic studies are based on assets and not just liability, integrated and system-based taxonomy has a good probability of expansive implementation in the future owing to its functionality. It may be a gradual change, but it surely helps in obtaining the bigger picture basically concatenating its genome to its state of being (phenotype) and also various environmental factors. Formulating virtual taxa related to taxonomy is on the other side a liability used only for aiding taxonomists to understand its inherent property of uncultivated state. It is a visualisation aid. Despite the fact that many methods have been realised and still being developed, there is still an undying need for the quick and rapid method of identification of bacteria.

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2 Bioinformatics Tools for Microbial Diversity Analysis

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Abstract

With several microbes discovered and rediscovered, there is a growing need to understand their lineage, origin, and occurrence. Given the microbial diversity and its importance in global impact, it would be interesting to explore the microbial resources to better disseminate the phenotyping, epidemiological investigations, screening, and metagenomics. There are several bioinformatics tools to mention from phylogenetic taxa, sequence and structural relationship, evolutionary mechanisms, horizontal gene transfer, and importantly functional genomics. Here we bring an overview of genomic tools that have aided identifying isolates, species, and subspecies of uncultured microorganisms and inferring their functional roles. While giving a gist of tools, we also discuss the features and limitations of these tools in the light of the emergence of next-generation sequencing (NGS) technologies.

Keywords

Microbiology · Bioinformatics tools · Functional genomics · Next-generation sequencing · Systems biology

2.1 Introduction

Microbial diversity analysis is fundamental to the field of microbial ecology, its function, stability, and productivity of a given ecological niche. Evolution plays a very important role in the dynamics of microbial diversity and is frequently

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considered when studying factors affecting the spatial as well as temporal diversity. In contrast to macroorganisms like plants and animals, the diversity matrices that are calculated for describing microbial diversity frequently use genomics-based technologies, specifically amplicon-based assessments that require some kind of "bioinformatics tools" beyond routine statistical tools. Integration of other omics-based information becomes essential while assessing functional diversity along with phylogenic diversity. The word "ome" means many, and the "omics" as a discipline has so far been aimed at resolving the complexity of living organisms. This has not only allowed to understand the life of the complex organisms but also identified the components associated with them besides ascertaining their association with the environment, thereby regulating the functioning of the system. As the era of "omics" is constantly generating huge amounts of biological data, bioinformatics as a branch of biology has gained immense importance over the years (Yadav 2015). Significant efforts have been made to improve various technologies related to studies of biota even as these efforts have led to the generation of different "omics" such as genomics, proteomics, transcriptomics, metabolomics, and so on. At the outset, it has been seen that single "omics" approach was not sufficient enough to characterize the complexity, and hence multi-"omics" approaches were used at all levels (Zhang et al. 2010). High-throughput genome sequencing has opened new perspectives for microbiology with beginnings in not only the development of numerous databases, web resources, and software, but also allowing the tools to be used for a wide array of applications (Singh et al. 2012). The development of bioinformatics approaches has enabled researchers to efficiently work on various aspects of microbiology such as diversity, molecular taxonomy, community analysis, pathogenesis, and secondary metabolism. The use of computational approaches to study biological information has been the major focus of bioinformatics. Leveraging knowledge underlying biology in combination with physical and chemical information is the core strength of bioinformatics. The revolutionary growth in computational competencies and the explosion of data derived from sequencing, etc., had allowed development of bioinformatics resources for core biologists with specializations like microbiology (Ussery et al. 2009).

On the other hand, with microbial genomes being easier to sequence compared to eukaryotic genomes, the goals such as development of rational drugs and antimicrobial agents, development and evolution of new bacterial strains, development of better vaccines, protein biomarkers, and better understanding of host-bacteria interactions have gained momentum with integration of bioinformatics techniques and databases (Bansal 2005). Bioinformatics can be used to analyze available experimental wet-lab data and perform mathematical and statistical modelling. Automation of genome sequencing, development of integrated genomic and proteomic database, identification of new potential gene functions, derivation and engineering of metabolic pathways, analysis of gene expression, and development of antimicrobial drugs are the major thrust areas for research. There are many different tools and databases that had been developed by the scientific community all over the worlds, and some of those resources are tabulated below (Table 2.1).

Table 2.1 Selected list of microbial resources

Microbial resource	URL[a]	Key features
IMG (Integrated Microbial Genomes)	https://img.jgi.doe.gov/	Annotation and analysis of microbial genomes and metagenomes
MicrobesOnline	http://www.microbesonline.org	Portal for comparative and functional microbial genomics
ModelSEED	http://modelseed.org/	Portal for curated genomic data and automated annotation of microbial genomes
GOLD (Genomes Online Database)	https://gold.jgi.doe.gov/	Resource for comprehensive information about genome and metagenome sequencing projects
CDD	https://www.ncbi.nlm.nih.gov/cdd	Conserved domain database
Pfam	https://pfam.xfam.org/	Database of protein families
STRING	https://string-db.org/	Database of protein association networks
Ribosomal Database Project(RDP)	https://rdp.cme.msu.edu/	16S rRNA gene database
SILVA	https://www.arb-silva.de/	rRNA gene database
GREENGENES	http://greengenes.lbl.gov/	16S rRNA gene database
Bacterial Isolate Genome Sequence Database (BigSdb)	https://pubmlst.org/software/database/bigsdb/	Bacterial isolate genome sequence database
EBI metagenomics	https://www.ebi.ac.uk/metagenomics/	Portal for submission and analysis of metagenomics data
EcoCyc	https://ecocyc.org/	*E. coli* genome and metabolism knowledge base
RegulonDB	http://regulondb.ccg.unam.mx/	*E. coli* transcriptional regulation resource
Pseudomonas Genome Database	http://www.pseudomonas.com/	Pseudomonas genome database
PATRIC	https://www.patricbrc.org/	Portal for many prokaryotic pathogens
EuPathDBs	https://eupathdb.org/	Portal for many eukaryotic pathogens
TBDB	http://genome.tbdb.org/tbdb_sysbio/MultiHome.html	Integrated platform for tuberculosis research
TCDB	http://www.tcdb.org/	Transporter classification database
TransportDB	http://www.membranetransport.org/transportDB2/index.html	Transporter protein analysis database
MetaCyc	https://metacyc.org/	Metabolic pathway database
Kyoto Encyclopedia of Genes and Genome	https://www.genome.jp/kegg/	Genome database with emphasis on metabolism
MiST	http://fgrtools.hms.harvard.edu/MIST/help.jsp	Microbial signal transduction database

(continued)

Table 2.1 (continued)

Microbial resource	URL[a]	Key features
SwissRegulon	http://www.swissregulon.unibas.ch/	Genome-wide annotations of regulatory sites in model organisms
RegPrecise	http://regprecise.lbl.gov	Database of regulons in prokaryotic genomes
Orione	http://orione.crs4.it/	Web-based framework for NGS analysis

Adapted from Zhulin (2015)
[a]All databases have been last accessed on August 3, 2018

2.2 Bioinformatics to Next-Generation Sequencing

Rapid and cheap sequencing methods had revolutionized the way the large chunks of genomic fragments are described. Not only the classical queries in the form of evolutionary status of a microbe or functional classification can be done via sequence analysis, but one can also predict routine diagnosis and public health care (Quainoo et al. 2017; Köser et al. 2012). One such functional classification for microbial communities is the sequence analysis using 16S rRNA (Langille et al. 2013). A wide gamut of applications has been dealt in the recent past in the areas of comparative genomics (Fraser et al. 2000), intraspecies diversity (Tettelin et al. 2008), development of specific databases (Michael B Prentice 2004), pathway analysis disseminating complex disease, microbial community modelling (Ramanan et al. 2012; Cardona et al. 2016), microbial evolution (Jungck et al. 2006), genome restructuring, and whole genome comparisons (Bansal 2005). Species taxonomy requires extensive integration of theories and methods from all the allied areas which study the origin, limits, and evolution of species. It uses a hierarchical system to categorize organisms. Due to the boom in sequencing data, a radical shift was seen toward DNA-based classification. This in turn led to the development of various tools for analyzing sequence information to taxonomical data. Phylogenetic trees are one of the major methods to identify DNA-based evolutionary relationships. The different tools used for evolutionary studies are given in Table 2.2.

2.3 Microbial Whole Genome Assembly and Annotation

Ever since the 454's NGS technology came up, a wide number of platforms in the form of Illumina, Ion Torrent, Pacific Biosciences, and Oxford Nanopore have flourished. Of them, Illumina in general is widely used, and arguably it has been used for short-read high-throughput sequencing with sufficient depth to accurately determine the sequence of a prokaryotic genome. However, as the genomes produce several sequences, collection of contigs and reads, assembling them, closing the gaps, and so on are manual and cumbersome steps. With whole genome reference assemblies available, there is a paucity of exact coverage/alignment mapped to them, thus paving way for de novo sequencing.

Table 2.2 List of important tools in phylogeny and their description

Database	URL and reference[a]	Key features
Clustal W	http://www.clustal.org/clustal2/ Thompson et al. (1994)	Clustal W is the command line version It is almost platform independent and removes duplicity in the alignment Uses progressive alignment algorithm that makes multiple alignment with each sequence in the set but does not use the entire information, so that remaining info go into waste It does not remove the gap between the alignments which in turn affects the output
Clustal X	http://www.clustal.org/clustal2/ Jeanmougin et al. (1998)	Poor alignments are highlighted, and it has the ability to improve and evaluate the quality of alignment It is based on the graphical version unlike Clustal W, which has command line interface If there is any gap in the ambiguous sequence, then the enormous amount of data used will go waste
MUSCLE	https://www.ebi.ac.uk/Tools/msa/muscle/	Hexamers are used to speed up the process of alignment and a more accurate distance measure Performs 2–3 iterations via tree-based partitioning of the sequences
Phylip	http://evolution.genetics.washington.edu/phylip.html Ahmed Mansour (2009)	Phylip is a standalone software and can be used on any platform The format requirements for Phylip are rather stringent, and any deviation will result in an error message "unable to allocate memory" and then the program terminates very slow; File renaming is difficult Still no: Codon model, Bayesian inference cannot read NEXUS standard files
T-coffee	http://tcoffee.crg.cat/ Notredame et al. (2000)	Provides a simple and flexible means of generating multiple alignments, using heterogeneous data sources. An optimized method, which is used to end the multiple alignment that best fits the pairwise alignments in the input library It is fast and relatively robust. It is composed of different tools and modules such as CORE, Mcoffee, and EXPRESSO for structure alignment, combining alignments, and evaluation It takes longer time to align multiple sequences than other programs. It has been cited in a limited number of peer-reviewed journals compared to Clustal W. however, this number is increasing rapidly every day
PAUP	http://paup.phylosolutions.com/ Matthews and Rosenberger (2008)	Has friendly graphical interface and supports both windows and Unix

(continued)

Table 2.2 (continued)

Database	URL and reference[a]	Key features
Trex	http://www.trex.uqam.ca/ Boc et al. (2012)	It is dedicated to the reconstruction of phylogenetic trees and network reticulation and to infer horizontal gene transfer (HGT) events
Phylogeny.fr	http://www.phylogeny.fr/ Dereeper et al. (2008)	A web service used for reconstruction and analysis of phylogenetic relationships
iTOL Interactive tree of life	https://itol.embl.de/ Lvica Letunic and Bork (2016)	Tool to view phylogenetic trees and manipulate them using various graphical formats
POWER	http://power.nhri.org.tw/power/home.htm	
PhyloWidget	http://www.phylowidget.org/ Jordan and Piel (2008)	It is used to visualize, modify and publish phylogenetic trees online and interface with databases. It is very speedy and responsive in tree rendering
OneZoom	http://www.onezoom.org/ Rosindell and Harmon (2012)	It displays phylogenetic trees using interactive fractal inspired graph (IFIG), which gives details in one click
EvolView	http://www.evolgenius.info/evolview/#login He et al. (2016)	It visualizes and manipulates phylogenetic trees using various formats. It also exports phylogenetic trees to different types of text like phyloXML, nhx, etc. organizes and adds datasets in an efficient way
TreeBASE	https://treebase.org/treebase-web/home.html	TreeBASE accepts various types of data like tree of genes, tree of population, tree of species. User-submitted phylogenetic trees and other data used to create them are deposited in TreeBASE
leBIBI[QBPP]	https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi Flandrois et al. (2015)	It is a set of web tools and database for analysis of prokaryotic sequences. It uses a wide range of databases covering different markers with various degrees of stringency. It also provides fully documented results
Tree of life web project	http://tolweb.org/tree/ David et al. (2007)	"The tree of life web project" (ToL) is a collaborative effort of biologists and nature enthusiasts from around the world
PhylOPDb	http://g2im.u-clermont1.fr/phylopdb/ Jaziri et al. (2014)	Resource for friendly web interface for phylogenetic oligonucleotide probe database to browse 16S rRNA-targeted probe
NCBI taxonomy	https://www.ncbi.nlm.nih.gov/taxonomy Wheeler et al. (2007)	"The taxonomy database is a curated classification and nomenclature for all of the organisms in the public sequence databases"

[a]All databases have been last accessed on August 3, 2018

2.3.1 Assembly

The sequences that produce very short reads (ca. <50 nt), particularly for de novo assembly of a whole genome, would have several abundant repeats and offer the lowest per-base cost approaches. So there is an immense challenge to consider the

best or optimal available read and further proceed for the best assembly. Whereas the aforementioned approach fits for eukaryotes, it is just beginning to understand how this varies among prokaryotes. Nevertheless, the sufficient depth surmounted by the coverage/depth, hashing and *k*-mer length obtained from de novo sequencing reads would decide the best fit. We highlight a few challenges that these statistical tools preferentially address.

1. *k*-mer counting of a read determines the frequencies for each fixed-length word of size *k*. The goals of these sub-sequences include data processing for de novo assembly, coverage estimation, and repeat detection. While short reads have sparsely mapped erroneous *k*-mers, only a small number of the total possible number of *k*-mers, for example, in Illumina, with a detectable 0.1–1% per-base error rate might generate unique mers. An unmet challenge is to check them for sequence analysis, and one needs to reach consensus using two or more tools. A host of tools, viz., Velvet, Kmergenie, and Minia, are known for checking these *k*-mer lengths, and emerging tools include Jellyfish, Tallymer, BFCounter, DSK, KMC, KAnalyze, and Turtle (Zhang et al. 2014).
2. Hash length/tables are based on the *k*-mer implementations in which the non-overlapping set of functionality stores the *k*-mer counts. While these counts are preferentially stored in the form of hash tables counting unique *k*-mers, KMC and KAnalyze are the best known tools to count them in low-memory false-positive and false-negative trade-offs (Roy et al. 2014). In addition, this can be overcome by measuring accurately the contig read lengths of $N_{50.}$

2.3.2 Annotation

In the recent past, prokaryotic genomes have grown exponentially, resulting in par with Moore's law in a huge reduction in the time and money investment per project (McLean et al. 2009). Many automated annotation pipelines do exist room for improvement as they poor annotation errors and inconsistencies with gene records. Featuring coding sites (CDS), ribosomal-binding sites (RBSs) and start/termination sites would give better prediction of strain-specific differences and homologs mapped (genetically) to its neighboring relatives. Fortunately, there are good annotated, if not curated, genomes available in gene banks which would perhaps avoid introduction of more errors, and probably these stringent requirements could be done to ensure acceptance of healthy genome annotation. One such pipeline that has been widely used is from GenBank's https://www.ncbi.nlm.nih.gov/genome/annotation_prok/. The current limitation still heralds these pipelines, for example, annotation records omitting the evidence tags and reliability of reference genome. A challenge would still remain to annotate multiple genomes at one go wherein we could assign the level of quality to annotation to be novel.

2.4 Microbial Pan-Genomics

The concept of the 'pan-genome,' a term coined by Tettelin et al. in 2005 can be surmised, as the entire genomic repertoire of a given species or a phylogenetic clade when systematics is employed for defining multiple species. Various strains of a bacterial species might differ substantially in their gene content. The total gene pool of a species might be orders of magnitude larger than the gene content of any single strain. A microbial pan-genome is composed of core genes or a core genome containing genes shared by all strains of dispensable genome containing accessory genes that conserved in two or more strains and genes unique to single strains (also known singletons). The core genes are responsible for the basic aspects of the biology of the species and its major phenotypic traits, while the accessory genes and singletons usually pertain to supplementary biochemical pathways and functions that may confer selective advantages such as ecological adaptation, virulence mechanisms, antibiotic resistance, or colonization of a new host. The pan-genome analyses represent a new approach to species definition and provide a framework for estimating and/or modelling the genetic diversity, dynamics, and evolution of microbial taxon (Tettelin et al. 2005). Pan-genomes are analyzed at species level and can be informative at any taxonomic level covering the entire bacterial domain.

A number of software packages and tools capable of clustering orthologous genes, identifying single-nucleotide polymorphisms (SNPs), constructing phylogenies, and profiling core/shared/isolate-specific genes have been developed. These have been reviewed by Xiao et al. (2015), and some of the popular tools and web servers dedicated to pan-genome analysis that offer the possibility to compute pan-genome analysis for genomes provided by a user are given in Table 2.3. Recently, a web server called PanGeneHome has been developed that offers a comprehensive and uniform framework using precomputed pan-genome analyses at a large scale for already sequenced genomes (Loiseau et al. 2017). About 2674 bacterial and 167 archaeal genomes, which have 8,912,641 protein-coding genes, were used to construct orthologous clusters (OC) and KEGG orthologous groups (KO) which are updated regularly. Analysis of any given number of species on this web server would result in pan-genome curves and matrices that throw light on the genomic plasticity and clade diversity of interest, as well as potential of core genes for the selected taxa.

2.5 Microbial Metagenomics

Analysis of genomic DNA from a whole community has been dubbed as metagenomics, which literally means "beyond the genome." This is different from genomics, wherein one is interested in the analysis of genomic DNA of an individual organism. Metagenomics can be understood as the random shotgun sequencing of microbial DNA, without selecting any particular gene, although in literature this term has also been used for those studies where only a part of 16S rRNA has been selected for analysis. The term first appeared in a study of soil microbes, and it has been employed in a variety of contexts including 16S rRNA gene diversity from an

Table 2.3 Selected list of pan-genome analysis tools

Database/tool	URL[a]	Key features
BPGA	http://sourceforge.net/projects/bpgatool/	Identifying variants and gene clustering, KEGG pathway, analysis
CAMBer	http://bioputer.mimuw.edu.pl/camber/index.html	Identifying variants
GET_HOMOLOGUES	http://www.eead.csic.es/compbio/soft/gethoms.php	Identifying variants, plotting homologs
Harvest	https://github.com/marbl/harvest	Identifying variants
ITEP	https://price.systemsbiology.net/itep	Identifying variants, plotting pan-genomic profiles, function-based searching
PanCake	https://bitbucket.org/CorinnaErnst/pancake/wiki/Home	Identifying variants
PanCGHweb	http://bamics2.cmbi.ru.nl/websoftware/pancgh/pancgh_start.php	Identifying variants, phylogeny
PanGP	http://PanGP.big.ac.cn	A visualizer and building pan-genomes
PANNOTATOR	http://bnet.egr.vcu.edu/pannotator/index.html	Identifying variants, function-based searching
Panseq	https://lfz.corefacility.ca/panseq/	Identifying variants, plotting pan-genomic profiles
PGAP	http://pgap.sourceforge.net/	Identifying variants, plotting pan-genomic profiles, function-based searching, clusters of orthologous gene (COG) analysis
PGAT	http://nwrce.org/pgat	Identifying variants, plotting pan-genomic profiles, COG analysis
ROARY	https://sanger-pathogens.github.io/Roary/MicrobialMetagenomics.docx	Plotting pan-genome profiles and gene clustering. A pipeline program
Spine and AGEnt	http://vfsmspineagent.fsm.northwestern.edu/index_age.html	Identifying variants

Adapted from Xiao et al. (2015)
[a]All databases have been last accessed on August 3, 2018

environment (Handelsman et al. 1998). Marker gene sequencing such as 16S rRNA does have some drawbacks in that it does not capture viruses. Recent studies also demonstrated that 16S or 18S rRNA gene-based amplicon sequencing missed out >50% organisms (Paez-Espino et al. 2016). Alternatively, metagenomics can be defined as the sequence-based, functional analysis of a collection of genomes (microbial) contained in a given environmental sample. The species community composition of an environmental sample is far complex, and the entire genome reconstructions would be a grossly simplified presentation of the actual complexity. To give a realistic estimate of community composition, it is estimated that marine

Table 2.4 A selected list of metagenomic tools used for metagenome assembly and annotation

Tool	URL and reference[a]	Key features
MIRA	https://sourceforge.net/projects/mira-assembler/files/MIRA/stable/ Chevreux et al. (2004)	Sanger, 454, Illumina, ion torrent, PacBio; reference based
AMOS and MetaAMOS	https://github.com/treangen/MetAMOS Treangen et al. (2013)	Genomic scaffolds, open reading frames, and taxonomic or functional annotations
SOAP	http://www.soap.genomics.org.cn Li et al. (2008)	Single-genome de novo assembly, reference-based assembly
ESOM	http://databionic-esom.sourceforge.net/ Dick et al. (2009)	Composition-based binning; binning
MEGAN	http://ab.inf.uni-tuebingen.de/software/megan6/ Huson et al. (2011)	Similarity-based binning; binning
WIMP	https://doi.org/10.1101/030742 Juul et al. (2015)	Complete metagenome pipeline allowing data collection from unprocessed samples to species/strain-level classification
Centrifuge	https://ccb.jhu.edu/software/centrifuge/ Kim et al. (2016)	Metagenome classification
Kaiju	Menzel et al. (2016)	Taxonomic classification

[a]All databases have been last accessed on August 3, 2018

communities are of the order of 100–200 species per milliliter of water. Soil is more complex, with approximately 4000 species per gram soil for estimated species richness. The initial metagenomics studies were for reconstructing the viral communities in oceans and human feces. These studies led to the critical observation that the gut is rich in Gram-positive bacteria and seawater in Gram-negative bacteria. The impact of these microbial genomes, studied through metagenomics, on human biology offers exciting prospects for scientific investigations toward betterment of human health and therapies (Riesenfeld et al. 2004). A collection of tools, which are popularly used for metagenome-based in silico analyses, is given in Table 2.4, but for an exhaustive list, refer to the review by Oulas and colleagues (Oulas et al. 2015).

- Identifying an ideal phylogenetic anchor is critical in establishing phylogenetic affiliation of the source of genes in a genome. One can proceed by identifying anchors on a DNA fragment and find fragments of genome linked to it with anchors.
- With an estimate of 200 species per ml, the metagenome would be of the order of 1 Gbp DNA. To get a good representation of rare members with other species, one needs to have a sequencing library with a minimum of 100-fold coverage. This in turn requires a large amount of DNA isolation and cloning.
- Insert sizes of sequencing libraries play an important role in capturing microbial diversity and complexity of communities comprising the metagenome. Smaller

insert libraries are convenient because lysing of microorganisms can be done using harsh methods.
- Rapid pace of "draft" genome sequencing has produced tens of thousands of new genomes, many of which are highly fragmented and incomplete.

2.5.1 Taxa Composition Quantification

Amplicon sequencing is routinely applied when marker genes are known, for example, one typically starts a metagenome study by sequencing the 16S rRNA gene (Tringe and Rubin 2005). The reads obtained from preprocessing the raw sequences of 16S can be mapped to a precomputed phylogenetic tree in two ways, with and without invoking dependence on taxonomy (Caporaso et al. 2010; Matsen et al. 2010). The method that works independent of taxonomy clusters the sequences into operational taxonomic units (OTUs). A software package, PPLACER, is suggested for this type of studies. It works in a classical way of using maximum likelihood and Bayesian probability for mapping reads to a predefined reference phylogenetic tree characterized by a fixed topology and branch length. The program calculates posterior probability of a read placement on an edge. The output would be a file with read counts for each of the edge intervals. Hence, one can order microbes with similar characteristics using phylogeny (Matsen et al. 2010). The alternative is to cluster the reads, at some similarity level (say 95–98%), into OTUs. Clustering is carried out using the classical method of computing pairwise Hamming distances. The method does not provide a precise characterization of species, yet it can be used to approximate the taxonomic rank of the species. A database with known bacterial 16S rRNA sequences can be employed for comparison and assignment of taxonomic lineage to each OTU. Genus-level aggregation of OTUs is quite a robust method and can be used to analyze the abundance levels (Hamady and Knight 2009).

2.5.2 Species Composition Quantification

Metagenome Analyzer (MEGAN) is a tool which can be used with a mixture of reads to infer microbial genomes (Huson et al. 2011). All the reads are subjected to a BLAST (Basic Local Alignment Search Tool) search against a collection of databases with known reads. The resulting hits are collected, and taxonomic IDs (taxon IDs) are assigned based on the National Center for Biotechnology Information (NCBI) taxonomy. The Least Common Ancestor (LCA) algorithm produces summaries of the results of read assignments at various levels of NCBI Taxonomy. Reads are annotated, based on the closest matching sequence. A word of caution is in place here; the best matching sequencing need not correspond to a related organism. To circumvent this issue, a tool was developed which addressed genome relative abundance and average size (GAAS) (Angly et al. 2009). This works in a very similar way to perform BLAST, wherein all significant hits (target genomes) are retained, for a given query sequence, and weights are assigned based on an expectation value as follows:

$$E_{ij} = m_i n 2^{-S_{ij}}$$

where i is the query sequence, j the target genome sequence, m_i effective query length and database size, and S_{ij} the HSP (high-scoring pair) bit score. The possibility of high local similarity with longer genomes led to a modification of E-value where n was replaced with effective length of the target genome t (t_{ij}) (Li 2015).

$$F_{ij} = m_i t_j 2^{-S_{ij}}$$

Weights can then be calculated easily as follows:

$$w_{ij} = \frac{\frac{1}{F_{ij}}}{\sum_j \frac{1}{F_{ij}}}$$

Hence, for each target genome, one gets:

$$W_j = \sum_j w_{ij}$$

Finally, one can calculate the relative abundance of the species as:

$$Ab_j = \frac{\frac{W_j}{t_j}}{\sum_j \frac{W_j}{t_j}}$$

It is recommended to use this tool through NCBI, and by default GAAS utilizes NCBI *RefSeq* as the target database (https://www.ncbi.nlm.nih.gov/refseq/).

The above approach, like any automated method, is prone to present erroneous results, which requires careful scrutiny. In particular, the possibility of identifying false species is very high in either of these approaches, and in addition the methods are computationally very intensive, as they involve read alignment to thousands of complete genome sequences of a large number of species. An approach to overcome this computational limitation involves alignment to marker genes, specifically, clade-specific marker genes. Based on the aligned reads to these marker genes, species abundances can be quantified. A tool which supports this formalism is available as metagenomic phylogenetic analysis (MetaPhlAn) (Segata et al. 2012). The tool acquires its backend databases and markers from integrated microbial genomes (IMG) system (https://img.jgi.doe.gov/). Additionally, a tool called GSMer has been developed which can use *k*-mers instead of coding genes to identify species-specific markers. In this approach, genome-wide *k*-mers are identified from the available microbial genomes, and these are then employed for species- or strain-level identification (Tu et al. 2014). Once the reads are mapped to markers, adjustments are made for marker length and the total number of mapped reads for calculating the relative abundances of the species. Care must be taken with the

assumption that the reads are uniformly distributed across different marker genes, as in practice this is not the case. One can attempt by modelling the read count data over multiple samples using a Poisson distribution.

2.5.3 Community-Level Analysis of Microbes

Summary of the microbial community is obtained by performing a community-level analysis, instead of attempting a taxonomy-level classification. An illustrative study has clearly highlighted the correlation between the human gut microbiome and metabolic markers. Hence, at the community-level, one needs to focus on diversity. Metagenome sequencing can provide species composition (as discussed earlier), which provides a way of measuring *within-sample diversity*, also known as *α-diversity* of the taxa. This diversity measure is quantified by calculating the Simpson index. The classical way of calculating the *Simpson* index is as follows:

$$D = \sum\nolimits_i^p \left(n_i \middle| \sum\nolimits_{j=1}^p n_j\right)^2$$

where $n_i$is the total number of reads of a particular OTU of species i and does not take into account the phylogenetic tree information. In simple terms, a phylogenetic tree can be envisaged to be composed of leaves (*current species*), internal nodes (*common ancestor*), and edges (lengths correspond to *evolutionary times*). Recently, a new definition for phylogenetic diversity (PD) was proposed as:

$$PD = \sum_i l_i g_\theta(D(i))$$

where l_i is the branch length of ith edge, $g(\theta)$ utilizes θ to modulate the effect of taxa frequencies, and $D(i)$ is the fraction of reads in the sample that are in leaves away from the root. The taxa frequencies are modelled using a function such as:

$$g(\theta) = [2 * \min(x, 1 - x)]^\theta$$

Here, $\theta = 0$ relates to classic PD, whereas $\theta = 1$ presents abundance-weighted PD.

We now address what is also known as Bray-Curtis dissimilarity, also known as α-diversity, for measuring the distance between two samples (metagenomes). It is defined by the following equation:

$$d_{XY} = \sum\nolimits_{j=1}^p {|n_{Xj} - n_{Yj}|} \Big/ {(n_{X+} + n_{Y+})}$$

where n_{Xj} is the taxa count in sample X and n_{Xj} is the total taxa count.

Let p_i^X and p_i^Y be the taxa proportions descending from branch i for communities X and Y, respectively. The UniFrac metric can be used to calculate both weighted and

Table 2.5 A selected list of tools for metagenome analysis

Tool	URL[a]	Key features
PHAST/ PHASTER	http://phaster.ca/	Useful in finding prophages in bacterial genomes
QIIME	http://qiime.org/	A pipeline for analyzing raw metagenome sequencing data. Support for generating publication-quality figures
MetaPhlAn2	http://segatalab.cibio.unitn.it/tools/metaphlan2/	A pipeline for profiling microbial composition. Depends on the curated MetaPhlAn2 database
StrainPhlAn	Open source http://segatalab http://segatalab.cibio.unitn.it/tools/strainphlan/	Strain-level resolution in profiling known microbial species. Depends on the curated MetaPhlAn2 database
MetaTrans		Tool for mapping taxonomic and gene expression analysis which also integrates quality control and rRNA removal
Tbooster	http://tbooster.erc.monash.edu	Ensemble methods for predicting effector proteins in bacterial systems, specifically for T3SS, T4SS, and T6SS

[a]All databases have been last accessed on August 3, 2018

unweighted distances. Community structure differences can be described using permutation-based non-parametric analysis of variance (PERMANOVA).

NGS revolutions, has imparted with an impact on every facet of biological sciences; microbiology and allied areas are no exception. The area has matured to an extent that the software tools employed for assembly and annotation have been standardized to an appreciable extent. The advent of metagenomics has greatly expanded the questionnaire of microbiologists. Gene expression levels of microbiota are addressed using meta-transcriptomics. Many tools are developed for downstream analysis of the sequencing data. The pace of tool development is also progressing so rapidly that it would not be surprising to see deployment of deep learning or AI-based strategies for metagenome analysis. It is difficult to list here every tool developed; hence a select set of general applicability is given in Table 2.5.

2.5.4 Metagenome Workflow

A common first step before submitting the data to any of the above-listed tools is to run a battery of computational tools for quality control. This is followed by identifying and removing low-quality sequences and contaminants. Some of the popularly used tools are given in Table 2.5, which include programs such as FastQC, BB tools, Cutadapt, and Trimmomatic. The features and sequence of steps followed in the workflow of MetaPhlAn v2.0 are given in Fig. 2.1.

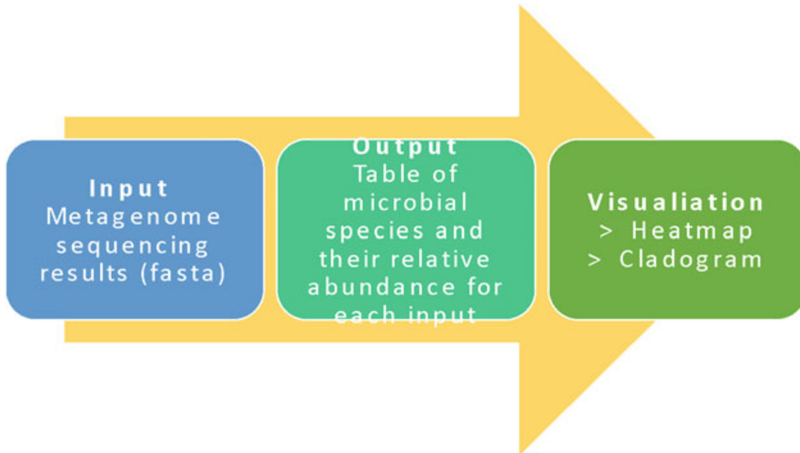

Fig. 2.1 Workflow for analysing 16srRNA in sequences using MetaPhlAn2

It is suggested to install MetaPhlAn latest version (version 2.0 as of now) by following instructions given at https://bitbucket.org/biobakery/biobakery/wiki/metaphlan2. The tool accepts input from a sequencing experiment in formats like .*fasta*, *.fastq*, *.sam*, and *.tar.bz*. Simply provide the type of input file and direct the output to a file. One can also run the program utilizing the multi-core architecture of the compute facilities, using the *nproc* parameter. One can then use the auxiliary visualization tools like *hclust2* to generate a heatmap for visualizing the results. Metagenomics data can also be analyzed using another popular tool QIIME. As described earlier, one may start by generating and picking OTUs. The input would be a file typically in FASTA format. A script called *pick_open_reference_otus.py* can be used for this purpose. Two options are provided here:

(a) De novo OTU picking—try this mode when you lack a reference database of sequences. This can be done by using the script *pick_de_novo_otus.py*.
(b) Regular OTU picking—should be run when comparing non-overlapping amplicons like V2 and V4 regions of 16S rRNA.

In the case of a closed-reference OTU picking, reads are clustered against a database, and the non-mapping reads are excluded from the further downstream analyses. This can be attempted using the script *pick_closed_reference_otus.py*. The default reference database is *Greengenes* OTUs (McDonald et al. 2012). One can find the version of this database by running the script *print_qiime_config.py*. The primary output of OTU picking exercise would be an *OTU table* which lists the OTU frequencies. The format of the table is according to the Genomics Standards Consortium Biological Observation Matrix (BIOM) standard format. The above commands also generate a phylogenetic tree where the tips are OTUs, which should

be used for downstream phylogenetic diversity calculations. One could run *summarize-table* command to generate summary statistics, taking care to extract the depth of sequencing information from this summary. The diversity analyses could be done with a script called *core_diversity_analyses.py*. The results are made available as *index.html*. The run with default parameters treats all samples as independent. Many times, one may be interested in categorizing samples using taxonomic summary or metadata, by using -c parameter. *SampleType* argument could also be specified in this case.

2.5.5 Recovering Genomes from Metagenomes

Recovering genomes with high fidelity from a metagenomics experiment (Sieber et al. 2018) involves handling high-complexity datasets, wherein binning the contigs has to be attempted using sequence compositional analysis. *Binning models* are constructed by employing sequence abundance and composition with marker genes from a reference database. Frequency of single-copy marker genes is used for assessing the quality of genome assembly in terms of completeness and contamination of predicted bins. The method has been implemented as a tool called as dereplication, aggregation, and scoring tool. It is an automated method that is intended to calculate a non-redundant set of bins from a single assembly. This tool can be thought of as a *meta-tool* that integrates various previously published approaches for *binning*. DAS tool employs four automated binning tools and runs with default parameters, for generating bins. The final bins obtained can be evaluated using CheckM (Parks et al. 2015), which is a set of tools used to assess the quality of genomes recovered from metagenome projects. It also needs to be supplemented with completeness and contamination check by using BUSCO (Simão et al. 2015). The scoring of each individual bin is based on single-copy gene (SCG) counts. The score is lower with an increase in duplicate SCGs per bin. It is expressed as in the equation below:

$$S_b = \frac{uSCG}{rSCG} - b\frac{dSCG}{uSCG} - c\frac{\sum SCG - uSCG}{rSCG}$$

This calculates a score based on the frequency of either bacterial or archaeal reference SCGs. The first term gives the fraction of SCGs present, where unique SCGs are in the numerator. Contamination is addressed and is useful in the event of duplicated SCGs (dSCG). The third term is described as a penalty term for mega bins, which is a ratio of extra SCGs to the total reference SCGs. The selection proceeds by initially selecting a redundant bin set, and the scoring function is used to calculate scores for bins. Then an iterative procedure is employed for generating a non-redundant set, with the extraction of the highest scoring bin. In case of matching bin scores, scaffold N50 is used to choose the best bin (the higher the better). All the contigs corresponding to the bins, which are excluded, are removed from all the bins. The iteration continues till the scores for every bin turn positive. Imperfect bins, and hence suboptimal genome assemblies, can be effectively addressed using this tool.

2.6 Multilocus Strain Typing

Multilocus sequence typing (MLST) is an important method to describe diversity of bacterial populations widely popular in epidemiology studies. Introduced to supplant the 16S rRNA-based descriptions (Maiden et al. 1998), it has become an important technique in classifying bacterial isolates into strains, especially in epidemiological context in case of pathogen outbreak surveillance. Conventionally, MLST involves PCR amplification of housekeeping genes followed by Sanger DNA sequencing. However, with the popularity of whole genome sequences, conventional MLST methodology is being replaced with a method based on next-generation (short-read) sequence data generated for whole genome sequencing. With the availability of whole genome data, MLST methods have evolved toward larger typing schemes, based on either a few hundred genes [core genome MLST (cgMLST)] or a few thousand genes [whole genome MLST (wgMLST)]. MLST can be derived from WGS using either de novo assembly/BLAST-based approach and/or mapping-based approach. De novo assembly/BLAST-based approaches work by assembling short reads into longer contigs and then comparing these contigs to a reference database using BLAST to assign an MLST type (Larsen et al. 2012). Mapping-based approaches align short reads to well-curated reference (allele) sequences representing all alleles from MLST loci using mapping tools such as BWA or Bowtie2. Mapping-based approaches allow the calculation of metrics for each allele to assess the quality of the match (Inouye et al. 2012). Generally, the mapping-based approach is more sensitive (Tewolde et al. 2016). A tool based on the synteny index (Anton Shifman et al. 2014), named Near HGT, has been described to measure the divergence of a gene from its native genomic environment. This method confirms candidate transferred genes based on the constant relative mutability that is a direct product of the Universal PaceMaker (UPM) of genome evolution phenomenon (Snir et al. 2012) to identify candidate HGT events between closely related strains (Adato et al. 2015). Some recent strategies have tried to avoid costly preprocessing of short-read datasets by working on the principle of k-mer indexing, which has been shown to be helpful in handling large short-read datasets in other bioinformatics contexts such as metagenomics. Two tools that follow this approach currently exist, stringMLST (Anuj Gupta et al. 2017) and StrainSeeker (Roosaare et al. 2017), although the latter assigns isolates to the nodes of a guide-tree, which is required prior to the typing phase. Lately, another tool based on k-mers has been described known as MentaLIST that does not require preassembled genomes, working directly with the raw WGS data, and also avoids costly preprocessing steps, such as contig assembly or read mapping onto a reference. MentaLIST uses an algorithm that follows the general principle of k-mer counting, introduced in stringMLST with some data compression improvements that lead to much smaller database sizes and a faster running time (Feijao et al. 2018). The novel feature of this tool is in the construction of colored de Bruijn graph for each locus/gene of a given MLST scheme, which allows the selection of a small subset of the MLST scheme k-mers, drastically reducing the size of the index file created and improving the running time at no precision cost. Various MLST tools have been reviewed previously (Page et al. 2017).

2.7 Secondary Metabolite Pathway Prediction

One of the most promising areas of research is prediction of secondary metabolite production since microorganisms are the major source for the novel natural products, which may serve as lead molecules for drug development. Genomics-based approaches offer complementary access to secondary metabolites' discovery through traditional screening-based approaches, by identifying secondary metabolite biosynthetic gene clusters (BGCs). It is now possible to assess the genetic potential of newly isolated producer strains and to identify previously unreported metabolites without spending much time in tedious screening using wet-lab methods. With the availability of a number of microbial whole genome sequences, it has been found that a high number of BGCs are commonly shared features among various taxa of bacteria though many remain cryptic for many reasons (Weber et al. 2015).

Before automated in silico tools became widely available, genome mining efforts have been performed by manually identifying key biosynthetic enzymes in genome data. For doing this, either the amino acid sequences of fully characterized enzymes were used as queries for BLAST or PSI-BLAST (Altschul et al. 1997), or—if alignments of a family of query sequences were available—these were used to generate profile hidden Markov models (pHMMs) which served as queries using software HMMer (Eddy 2011). BCGs were then identified by analyzing the upstream and downstream of the hit sequence and including encoded genes into the cluster. This kind of manual approach is still relevant for identifying BCGs, which are not yet uncovered by the automatic tools or where new prototypes have been described recently.

There are two important strategies in the implementation of automatic bioinformatics tools for in silico prediction of secondary metabolite BCGs with high precision (Weber and Kim 2016). The first approach is rule-based in that a given tool would identify genes encoding conserved domains in key enzymes that have associated roles in reactions such as the condensation (C), adenylation (A), and peptidyl carrier protein (PCP) domains of non-ribosomal peptide synthetases (NRPSs). Then these predefined rules are used to associate the presence of such hits with defined classes of secondary metabolites. More complex rules may take into account whether specific genes are present in close proximity or not. Such rule-based search strategies are implemented as one of the options in the pipeline—antibiotics and secondary metabolite analysis shell (antiSMASH). Similarly, clusters containing modular polyketide synthase (PKS) or NRPS genes could be detected by scanning the genomes or metagenomes for sequences that encode their characteristic enzyme domains and using tools such as NaPDoS, NP.searcher, GNP/PRISM, and SMURF in addition to antiSMASH (Blin et al. 2017; Zeimert et al. 2012; Li et al. 2009; Skinnider et al. 2015; Khaldi et al. 2010). However, these algorithms were unable to detect novel pathways that may use different biochemistry and enzymes as they are based on the prerequisite to have defined rules. Thus, the second strategy is to employ rule-independent algorithms, which are less biased. Examples of such tools are ClusterFinder and EvoMining, which use machine learning (ML)-based approaches or automated phylogenomics analyses to make their predictions

(Cimermancic et al. 2014; Cruz-Morales et al. 2015). When combined with existing cheminformatic tools, the abovementioned genomic data mining tools could become a game changer for the field of synthetic biology. As these computational approaches are rather new and dynamic, a web portal called SMBP has been created by Weber and Kim (2016) along with links to references and websites of tools and databases to help the research community (http://www.secondarymetabolites.org).

2.8 Predicting Horizontal Gene Transfer Events

Horizontal gene transfer (HGT) has been regarded as an important mechanism in microbial evolution and adaptation. HGT is an important mechanism that operates in organisms such as bacteria to directly acquire genetic material from a distantly related species. This way, donor bacteria can transfer new traits such as antibiotic resistance or pathogenic toxins to the recipients. By means of HGT, complete genes and functional units, known as insertion sequences (IS) or genomic islands (GIs), could be incorporated into the recipients' genome. A number of pipelines have been developed to identify HGT events in draft or completed genomes of bacteria. These can be broadly classified into two groups: parametric and phylogenetic methods. Parametric methods search for sections of a genome that significantly differ from the genomic average, such as GC content or codon usage (Jeffrey and Ochman 2002). Phylogenetic methods look at evolutionary histories of genes involved and identify conflicting phylogenies. Phylogenetic methods can be further divided into those that reconstruct and compare phylogenetic trees explicitly and those that use surrogate measures in place of the phylogenetic trees (Bansal et al. 2012; Christophe Dessimoz et al. 2008). These techniques could be used only if there is availability of complete and fully annotated genomes with sufficiently large differences that allow detection of HGT. In contrast, short reads obtained through NGS could also facilitate rapid analysis of unknown pathogens in silico. One such tool, Daisy, allows HGT detection directly from NGS data without requiring a de novo assembled genome. It determines HGT boundaries with split-read mapping and evaluates candidate regions relying on read pair and coverage information (Song et al. 2017; Kathrin Trappe et al. 2016).

2.9 Viriome Analysis

Viruses have also been known to play critical roles in shaping the composition and function of environmental microbiomes (Rohwer and Thurber 2009). Viruses were largely missing from systematic surveys of microbial diversity till recently, as they do not have universally conserved genes such as the 16S and 18S rRNA genes. However, > 125,000 new DNA viruses have been discovered by using the metagenomic technologies and also from mining the publicly available metagenomic datasets (Paez-Espino et al. 2016). It can be safely concluded that an explosion of viriome studies is round the corner with recent repurposing of tools

developed for bacterial genomes to viral genomes too. Strategies to identify and annotate viruses differ among different tools that range from analyzing marker genes, binning sequences or reads into taxonomic groups, assembling sequences into contigs, and then annotating the genes from the contigs for taxonomy to directly aligning short reads to a reference database and inferring virus types and abundances based on the alignment results. A simple and straightforward approach for virus taxonomic annotation is to align short reads to a marker gene database and identify viruses based on the alignments; for example, MetaPhlAn2 (Truong et al. 2015) uses this approach. However, this strategy does not work well when the input data contain species whose marker genes are not present in the database. Assembling short reads into contigs and then performing the analysis with them would produce more accurate results (Roux et al. 2017), though read assembly is very compute-intensive and time-consuming for large metagenomic datasets and could also generate chimeras. To overcome this drawback, another set of tools such as MG-RAST (Meyer et al. 2008), ViromeScan (Rampelli et al. 2016), VIP (Li et al. 2016), and HoloVir (Laffy et al. 2016) could be employed, which directly align the reads to a reference database of whole genomes for taxonomy annotation. These tools were initially developed for bacteria but were repurposed for viruses. A recently developed tool FastViromeExplorer uses kallisto (Bray et al. 2016), a pseudoalignment-based approach developed for RNAseq data instead of the traditional read alignment tools such as BLAST or Bowtie2 used in earlier developed tools. FastViromeExplorer first uses kallisto to rapidly map short metagenomic reads to a reference virus database, then filters the alignment results based on minimal coverage criteria, and reports virus types and abundances along with taxonomic annotation (Tithi et al. 2018). FastViromeExplorer can detect both viruses and bacteriophages depending on the reference database deployed, which also becomes its limitation in that it cannot identify a virus or a bacteriophage if a related sequence is not present in the reference database, highlighting the need for improving the current viral sequence databases.

2.10 Conclusions and Future Directions

Though most of the tools described above exclusively use high-throughput short-read sequencing, third-generation single-molecule sequencing platforms like PacBio and MinION are fast catching up for microbial studies (Nakano et al. 2017). Their base-calling error rates are higher, and the throughput low limits their exclusive use in sequencing-based applications. Sequencing of genomic regions such as high/low GC content and tandem repeats poses a challenge for short reads, but the long-read technologies have not shown such systemic errors other than high error rates that are evenly distributed. However, using them in combination with short reads and performing a hybrid analysis are fast catching up as a standard approach (Madoui et al. 2015). Many tools such as MEGAN are being repurposed for this job or

standalone methods are being developed. The ability to classify sequences to the genus or species level is a function of read length, quality of the sequence, and reference database. High-quality short reads from MiSeq or ion Torrent use partial sequence of 16S rRNA gene, generally the V4 region or V1–V2 regions but rarely full-length gene sequence. Third-generation single-molecule DNA sequencers such as PacBio or MinION of Oxford Nanopore Technologies overcome the length limitation. Obviously, long reads in the range of >5 kb are advantageous in all kinds of analyses described above.

Despite impressive technological advancement in sequencing of both short reads in bulk and long single molecules of DNA, it is still not possible to accurately and unambiguously find the sequence from one end of the chromosome through to the other of a given organism, so that we find all the phylogenetic and functional information about a microbial species in its ecological niche. As Studholme (2016) pointed out, what we find is a model, a hypothesis, and our best guess at the biological reality, and there are choices to be made at each step of bioinformatics analysis, and each decision may impact the final result. It needs to be kept in mind that even starting from the same raw sequence data, it is perfectly possible to generate different end results by choosing different tools, different pipelines, different options, and parameter values or by different quality-control filtering regimes.

The most commonly used reference genome databases are the complete and draft genomes at NCBI, which has approximately 141,000 prokaryotic genomes that are currently available in the public domain, and the number is growing exponentially. With the help of such massive datasets, genome plasticity estimations through pan-genome analysis are helping in understanding evolution of prokaryotes and delineating species boundaries. Now that full genome sequences of even closely related organisms are becoming available, the microbial diversity analysis utilizing whole genomes could unearth hitherto unknown amount of variation in microbial communities in every ecological niche. Another development that could further revolutionize the microbial diversity studies and uncover the "Microbial Dark Matter" is in the area of single-cell genomics. Microbial single-cell genomics has been used to get insights into the functional diversity, interactions, and evolution of the yet uncultured microorganisms from marine and agricultural soils. By circumventing the need for arbitrary taxonomic binning as in the case of metagenomic assemblies, microbial single-cell genomics improves our understanding of microbial microevolutionary processes and helps calibrate the performance and interpretation of community omics tools. Direct comparative methods can accomplish analogously, but more precise results are possible in silico, wherever whole genome sequences are available for the community members of any ecological niche. Truly exciting and challenging times are ahead!

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3 Application of Genomics to Understand the Pathogenic Microbial Diversity

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Abstract

Conventional bacteriological, biochemical, and molecular methods used in diagnostic laboratories for identification of pathogens are laborious, time-consuming, and expensive. Moreover, it provides limited information which is not sufficient to evaluate the disease outbreak and epidemiological investigations. Due to drastic reduction in sequencing cost concomitant with an increase in the sequence quality, whole genome sequencing of prokaryotes based on NGS is economically feasible as a routine tool for clinical diagnostics and surveillance of pathogens. Availability of comprehensive biological information databases and advanced bioinformatics tools for analysis of pan-genomes, single nucleotide polymorphisms, virulence factors, antibiotic resistance, recombination, and lateral or horizontal gene transfer events have greatly facilitated the identification of emerging pathogenic strains, understanding their population dynamics, genomic plasticity, virulence and pathogenicity, and epidemiology. Genomics studies have greatly enriched our knowledge of various genetic events that have shaped pathogenic bacterial genomes and guided their evolution, such as mutations, insertions, deletions, duplications, inversions, transpositions, and recombination. Whole genome analyses of the classical mammalian *Bordetella* spp., *Vibrio cholerae*, and *Salmonella enterica* have revealed important features of their virulence and pathogenicity, as well as their evolution as successful human pathogens. Gene inactivation, polymorphism, accumulation of IS elements, and genome decay have guided the evolution of the classical *Bordetella* spp. as separate host-restricted pathogens, while the acquisition of pathogenicity islands by HGT mechanisms has the greatest impact on the evolution, virulence, and pathogenicity of *Vibrio cholerae* and *Salmonella enterica*.

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Keywords
Genomics · Pan-genome · SNPs · Virulence factors · Antibiotic resistance · Pathogenicity · Recombination · Horizontal gene transfer · PAIs · *Bordetella* · *Vibrio* · *Salmonella*

3.1 Introduction

At present, next-generation sequencing (NGS) technologies have emerged as the most attractive approach in studying genomics of pathogenic bacteria (Deneke et al. 2017). Genome of an organism represents the full complement of genes contained in the DNA of its chromosome(s) and a blueprint of physiological and metabolic functions (Narihiro and Kamagata 2017). With the expansion in sequencing approaches and sequencing of bacterial genomes, the repositories of biological sequences are getting significantly enriched very rapidly (Land et al. 2015; Vernikos et al. 2015). Whole genome sequences of bacterial pathogens are readily available and freely accessible from several biological databases that have driven microbiologists' interest toward the field of comparative genomics since it facilitates understanding of the basic biology, evolution, ecology, and genetic diversity (Land et al. 2015, Vernikos et al. 2015). Bacterial genomes are dynamic, vary in their composition and organization, and are shaped over millions of years by various genetic mechanisms such as deletions, insertion, duplications, inversions, transpositions, and recombination that cause chromosomal rearrangements due to the evolutionary forces of adaptations (Lawrence and Hendrickson 2005, Ochman and Davalos 2006). Genomic analyses have revealed intraspecies variations by refining the essential genes that are strain- or species-specific and expanded the total identified gene diversity within a species (Mazumder et al. 2005). The study of genomics has also become an essential tool to identify genomic signature accountable for virulence in pathogenic bacteria. Genome-scale variations among strains from the same pathogenic bacterial species have provided information critical to understanding of their ecology, evolution, host adaptations, virulence, and pathogenesis (Lasken and McLean 2014). The most discriminating feature between the pathogenic and nonpathogenic bacteria is the absence of virulence genes in the nonpathogenic microbes. Pathogenic capability of a bacterium relies on its virulence factors which can create infection and assist sustenance of bacterium in the hostile environment, leading to cause disease in a host body. Some phenotypic traits are mandatory to become a successful pathogen, such as virulence factors, antigenic determinants, and antibiotic resistance for the colonization in a host body (Peterson 1996). The virulence genes reside in the plasmid, pathogenic island, and chromosome of the pathogenic bacteria (Hacker et al. 1997). Pathogenic microorganisms acquire virulence traits during their transition from a free-living to host-adapted lifestyle where they face increasingly difficult and hostile host immune defenses (Aujoulat et al. 2012). However, the reasons for acquisition of virulence traits by

some otherwise commensal microbes are not clear, probably acquired by recombination or horizontal gene transfer (HGT) during intra- or interspecies interactions within specific host niche. These acquired virulence traits may, in turn, help the microbes to adapt to a within-host from an extra-host lifestyle, as well as help in their transmission from host to host during outbreak. The gene pool for virulence factor in most of the pathogens is not static and differ in combination, organization, and expression patterns of virulence factors that results in various clinical symptoms of infections (Pallen and Wren 2007). Gene acquisition through horizontal gene transfer seems to have the maximum influence on the ecological fitness of pathogenic bacteria by conferring novel metabolic capacity, such as acquisition of antibiotic resistance genes and virulence factors (Donkor 2013, Forde and O'Toole 2013). However, gene loss is also a critical phenomenon for bacterial pathogenesis, prominently for those genes contributing to surface antigen production (Moran 2002). Timing and the scale of genetic changes in the genome of the pathogenic species reveal different kinds of genome dynamics of the pathogen (Raskin et al. 2006). Virulent genes always encounter the selection pressure of host's internal environment like host immune response which results in the development of genetic signature in the putative virulence gene that can be used in its identification (Pallen and Wren 2007). Pathogenicity-specific genetic signature, functions, and characteristics of a gene can be identified based on comparative genomic approach. The genomic approach has revolutionized the process of understanding how the pathogenesis develops in bacteria. There are some obligate intracellular bacteria that cannot be maintained in vitro; in such cases, comparative genomics is the only method to identify virulence factors (Raskin et al. 2006). The genomic approach also allows characterization and identification of pathogenic properties in both culturable and non-culturable bacteria. The comparative genomic analysis combined with pan-genomic approach has opened the arena of pathogenomic studies (Rouli et al. 2015). Unlike targeted investigations of candidate genes, genome-scale methods have permitted the interrogation of full genomic repertoire of a population and have enabled understanding of the population structure, epidemiology, antimicrobial resistance mechanisms, and virulence, as well as the discovery of critical mutations and genetic mechanisms that guide host-microbe and microbe-microbe interactions in their natural environment, thereby greatly expanding our knowledge of bacterial lifestyle (Hibberd 2013). Genomic approaches for the identification of putative virulence factors facilitated further investigation of their function and mechanism using structural biology and other experimental methods, which in turn will aid in the design and development of novel drugs and vaccines to prevent infectious bacterial diseases.

3.2 Methods of Genomic Data Analysis

With recent advancements in the NGS technologies such as the Illumina MiSeq, Ion Torrent PGM, SOLiD, Roche 454 FLX+, Oxford Nanopore, or Pacific Biosciences, prokaryotic genome sequencing is possible in a short span in many labs. The main

goal of whole genome sequencing (WGS) is to extract the taxonomic identity and functional profile of an organism; however, challenges are still there in analyzing the genomic sequence data. Numerous software packages and web resources are available for WGS data analysis (Table 3.1). The most basic method for assigning a taxonomic or functional label to a genomic sequence is the BLAST program (Altschul et al. 1990, 1997) which can classify a sequence by finding the best alignment to a large database of genomic sequences. Besides this, there are several web-based publicly available genome annotation and analysis programs, such as NCBI-PGAP, RAST, IMG/M, and PATRIC (Table 3.1), which utilize sequence alignment and machine learning techniques to improve upon BLAST's accuracy. For the extraction of comprehensive functional information from genome sequences, these annotation and classification tools rely on curated databases, such as National Center for Biotechnology Information (NCBI) non-redundant (NR), SEED, Kyoto Encyclopedia of Genes and Genomes (KEGG), clusters of orthologous groups (COGs), protein families (Pfam), and others.

The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) is an automatic prokaryotic genome (chromosomes and plasmids) annotation pipeline that combines ab initio gene prediction algorithms with homology-based methods (Tatusova et al. 2016). It is capable of annotating both complete genomes and draft WGS genomes consisting of multiple contigs. The Joint Genome Institute's (JGI) Integrated Microbial Genomes and Microbiomes (IMG/M) system serves as a community resource for annotation and comprehensive comparative analysis of prokaryotic genomes by integrating all publicly available drafts and complete genomes in its database (Markowitz et al. 2012). It provides tools and viewers for analyzing and reviewing the annotations of genes and genomes in a comparative context. RAST (Rapid Annotation using Subsystem Technology) is a fully automated pipeline for annotating complete or nearly complete bacterial and archaeal genomes (Overbeek et al. 2014). For a new genome, the RAST annotation engine allows gene calling and their annotations by comparison to the FIGfam collection (protein families). The pipeline identifies protein-encoding rRNA and tRNA genes, assigns functions to the genes, predicts SEED subsystems to reconstruct the metabolic network from the newly sequenced genomes, and supports comparative analysis with the annotated genomes maintained in the SEED environment (Aziz et al. 2008). The web-based PATRIC (Pathosystems Resource Integration Center) platform provides a comprehensive collection of genomic data and a suite of analytic tools for genome assembly, annotation, comparison, and visualization of results to aid studies on bacterial pathogens (Wattam et al. 2017). The genomes in PATRIC are sourced from public databases and are consistently annotated using the RAST server. PATRIC integrates several types of data, including sequence typing data, genes essential for bacterial survival, antibiotic resistance, drug targets, transporters, virulence factors, human gene homologs, genomes, genomic features, pathways, protein families, and transcriptomics data.

The NCBI-NR protein sequence database (Entrez Sequences Help 2010) provides a non-redundant collection of protein sequences (i.e., all protein products encoded by one gene in a given species are represented in a single record) sourced from

Table 3.1 Software and web resources for genomic data analysis and visualization

Software/tool	Application/description	URL
NCBI Prokaryotic Genome Annotation Pipeline (PGAP)	Genome annotation (both chromosomes and plasmids)	https://www.ncbi.nlm.nih.gov/genome/annotation_prok/
IMG/M	Annotation and comparative analysis of microbial genomes	https://img.jgi.doe.gov/cgi-bin/m/main.cgi/
RAST	Bacterial and archaeal genome annotation	http://rast.nmpdr.org/rast.cgi/
PATRIC	Pathogen genome assembly, annotation, comparison, and results visualization	https://www.patricbrc.org/
Artemis Comparison Tool (ACT)	Pairwise comparisons between two or more DNA sequences	https://www.sanger.ac.uk/science/tools/artemis-comparison-tool-act
PSAT	Investigation of homologs and their neighborhoods in multiple genomes	http://www.nwrce.org/psat
BRIG	Circular comparative genome data visualization	https://sourceforge.net/projects/brig/
progressiveMauve	Multiple genome alignment, identification and visualization of SNPs, and rearrangements	http://darlinglab.org/mauve/
Artemis and Artemis Comparison Tool (ACT)	Alignment, comparison, and visualization of multiple genomes	https://www.sanger.ac.uk/science/tools/artemis-comparison-tool-act
CLC Genomics Workbench	Comparative genome analysis and visualization	https://www.qiagenbioinformatics.com/
PanOCT	Pan-genomic identification of orthologous proteins	http://panoct.sourceforge.net/
Panseq	Determination of core and accessory genomic regions	https://github.com/chadlaing/Panseq
kSNP3	Detection of genome-wide SNPs and phylogenetic analysis	https://sourceforge.net/projects/ksnp/files/
Samtools	Detection of SNP and short indel sequence variants	http://www.htslib.org/
EnteroBase	Analysis and visualization of genomic variation within enteric bacteria	https://enterobase.warwick.ac.uk/
BIGSdb	Multilocus sequence typing (MLST) for analysis of genetic relatedness among strains	https://pubmlst.org/software/database/bigsdb/
MentaLiST	Multilocus sequence typing (MLST) for analysis of genetic relatedness among strains	https://github.com/WGS-TB/MentaLiST
MLST	Multilocus sequence typing for characterizing strains	http://www.mlst.net/

GenPept, UniProtKB/Swiss-Prot, Protein Information Resource (PIR), Protein Research Foundation (PRF), Protein Data Bank (PDB), and NCBI RefSeq databases. Comprehensive functional categories facilitated in SEED (Overbeek et al. 2005) and KEGG (Kanehisa and Goto 2000) databases allow comparative genomics and metagenomics tools to highlight functional features represented by an individual organism or a whole community. The SEED and KEGG classification tree arranges metabolic pathways into a hierarchical structure in which all of the genes required for a specific function are arranged into subsystems and pathways, respectively. The COGs have been used for functional classification of proteins, particularly in microbial genome sequencing projects. The COG database (Tatusov et al. 2001) provides 17 functional categories for orthologous groups in order to facilitate functional studies and serves as a platform for functional annotation of newly sequenced genomes and studies on genome evolution. Pfam database (Finn et al. 2014) is a comprehensive collection of protein domains and families, represented as multiple sequence alignments and as profile hidden Markov models (HMMs) for the accurate classification of genomic sequences. The profile HMM is searched against a large sequence collection, based on UniProt Knowledgebase (UniProtKB) [UniProt Consortium 2012].

Bacterial genomes are characterized by large-scale rearrangements of DNA regions caused by multitude of mutational forces, including massive insertion-deletions, inversions, and transpositions of blocks of DNA from one part to another part of the genome, as well as due to duplication and/or loss of DNA fragments, and are especially prominent in bacterial pathogens. Therefore, it is important to visualize genomic comparisons to understand and interpret the combined effects of gene gain or loss and rearrangements within bacterial species. For this, there are several publicly available software tools that combine data analysis and visualization, such as BRIG, progressiveMauve, and ACT (Table 3.1). BLAST Ring Image Generator (BRIG) is an application that allows comparisons and visualization of multiple prokaryote genomes using BLAST (Alikhan et al. 2011). It generates comparative circular images showing similarity between a central reference sequence and other sequences as a set of concentric rings. It can show information derived from draft genome data, including contig boundaries, read coverage, or read mapping data. Also, it can display the presence, absence, or variation of a user-defined set of reference sequences in multiple datasets simultaneously, including unassembled next-generation sequencing reads. The progressiveMauve (Darling et al. 2010) is a Java-based tool for multiple alignment and visualization of closely related bacterial genomes that have undergone substantial amounts of gene gain and loss for analysis of genome evolution. It employs an alignment algorithm based on sum-of-pairs breakpoint score, which facilitates accurate detection of genomic rearrangements (Darling et al. 2010). It can accurately align regions conserved in subsets of genomes called locally collinear blocks (LCBs) which can be used to characterize the shared (core) and total (pan-genome) genome in multiple strains of a bacterial species (Darling et al. 2010). It identifies and aligns both conserved regulatory regions and

hypervariable intergenic regions. It can also be used to visualize the extent of local conservation of nucleotide sequences and identify point mutations or single nucleotide polymorphisms (SNPs) useful for downstream phylogenetic or evolutionary analyses. Artemis Comparison Tool (ACT) is a Java application for visualization of pairwise comparisons between multiple genomes (Carver et al. 2005). It allows simultaneous identification and analysis of regions of similarity and differences between genomes and exploration of conservation in DNA sequences of syntenic blocks, their annotation, and genetic variations (Carver et al. 2008).

3.3 Genomics Concepts for Identification and Characterization of Pathogens

3.3.1 Elucidation of the Pan-genome for Analyzing Pathogenic Bacteria

The size and gene content of bacterial genomes are reflection of their metabolism, pathogenicity, physiology, and lifestyles (Mazumder et al. 2005; McCutcheon and Moran 2012). Obligate intracellular bacteria (i.e., symbionts and parasites) often have smaller genomes with fewer genes compared to their nonpathogenic or free-living ancestors (McCutcheon and Moran 2012; Merhej et al. 2013). The "pan-genome" is defined as the additive gene repertoire within a given phylogenetic clade or species and comprises three different gene groups: the "core genes" that are common to all strains under study, "accessory genes" that are present in few stains, and "unique or strain-specific genes" that are present in a single strain (Tettelin et al. 2005). The pan-genome scheme allows prediction and modeling of genomic diversity within a species (Vernikos et al. 2015). This scheme can also be used as a tool for the identification and characterization of pathogenic strains. With the advent of pan-genomic studies, several automated tools have been developed (Table 3.1), such as the Pan-genome Ortholog Clustering Tool (panOCT) (Fouts et al. 2012) for pan-genomic analysis of closely related prokaryotic species or strains that can be used for accurately clustering orthologous proteins using a combined conserved gene neighborhood and homology search method. Another pan-genome sequence analysis program, the Panseq (Laing et al. 2010), is also used for detecting unique regions, as well as identify SNPs in core and accessory genome. Analysis of pan-genome provides information on all the mobile genetic elements and the resistance genes of a genome. Toxin/antitoxin (TA) genes that are small genetic elements have a role in superintegron stabilization which encodes proteins for adaptive functions like resistance and virulence (Socolovschi et al. 2013; Georgiades and Raoult 2011). Toxin/antitoxin (TA) genetic elements were also associated with the pathogenicity of some bacteria. It has been reported in most of the pandemic bacteria for humans (Fouts et al. 2012). Thus, pan-genome analysis is emerging as a major approach for detection of pathogenic microbes (Rouli et al. 2015).

3.3.2 Detection of Pan-genome Single Genome Polymorphisms for Pathogenic Bacterial Strain Identification

Generally, genetic variations among individuals within a population are regarded as the beginning point for adaptation and evolution among organisms (Morris et al. 2002). Spontaneously appearing single nucleotide polymorphisms (SNPs) is an important mechanism of genetic variation (Barrick et al. 2009). The pan-genome SNPs are referred to as the total SNPs present in genomes of distinct bacterial strains and provide information on genomic variation that appears in a species under the evolutionary forces of adaptation (Barrick et al. 2009). Often, different pathogenic strains exhibit variable ecological fitness, capacity to cause disease or virulence potential, and physiological host range (van Baarlen et al. 2007). Therefore, identification of the pan-genome SNPs is useful for strain identification, phylogenetic or evolutionary analysis, nosocomial transmission or epidemic disease outbreak investigations, source tracking, as well as identifying genotypes important for virulence and antibiotic resistance phenotypes (Gardner and Hall 2013). There are several pan-genome SNPs calling tools (Table 3.1), such as the progressiveMauve (Darling et al. 2010) which is a Java-based tool that detects pan-genome SNPs based on multiple genome alignment, and the kSNP3 program that identifies pan-genome SNPs and estimates phylogenetic trees without genome alignment or reference genome (Gardner et al. 2015).

3.3.3 Bacterial Virulence and Pathogenicity

Virulence factors (VFs) are gene products that are either secreted to the bacterial cell surface or released into the extracellular environment and increase the ability to cause infection or pathogenicity (Korves and Colosimo 2009). VFs are involved in the evasion of host immune defenses, colonization, toxicity, invasion, and transmission from host to host, are also responsible for antibiotic resistance, and promote intercellular communication (Wu et al. 2008; Korves and Colosimo, 2009). These molecules include proteins that aid in bacterial attachment to host cells or tissues (adhesins); molecules that disrupt physiology and kill host cells (toxins), molecules that suppress immune defenses (effectors); extracellular enzymes that break down host tissues (invasins), autotransporters (ATs), and secretion system proteins (type I to VI); polysaccharide capsules that resist phagocytosis and host immune defenses; and iron uptake molecules (siderophores) [Wu et al. 2008; Korves and Colosimo 2009]. Discovering VFs is the key to understand pathogenesis of bacterial diseases and for identification of targets for design and development of novel drugs and vaccines. There are several VF databases (Table 3.2) and bioinformatics methods available (Table 3.2) for the identification of virulence genes (VGs) that encode VFs which include sequence-based and interaction network-based methods. The Virulence Factor Database (VFDB) is one of the most widely used databases where the information for VFs is subcategorized according to their mechanism of action (Chen et al. 2016). VFs

Table 3.2 Tools and database related to virulence factor detection

Software/tool	Application/description	Database URL
ARG-ANNOT	Detection of existing and putative new ARGs	http://en.mediterranee-infection.com/article.php?laref=283%26titre=arg-annot
ResFinder	Identification of acquired ARGs	www.genomicepidemiology.org
VirulenceFinder	A tool for detection of VFs	https://cge.cbs.dtu.dk/services/VirulenceFinder/
VICMpred	A tool for prediction of VFs	http://www.imtech.res.in/raghava/vicmpred/
VirulentPred	A tool for prediction of VFs	http://bioinfo.icgeb.res.in/virulent/
ARDB	Identification of ARGs in newly sequenced genomes	https://ardb.cbcb.umd.edu/
VFDB	Database of bacterial VFs and virulence-related genes	http://www.mgc.ac.cn/VFs/
CARD	Identification of ARGs in newly sequenced genomes	https://card.mcmaster.ca/
MvirDB	Database of microbial protein toxins, VFs, and ARGs	http://mvirdb.llnl.gov/
PARTIC_VF	Database of curated VFs from pathogenic bacteria	https://patricbrc.org/view/SpecialtyGeneList/? and (eq(source,PATRIC_VF),eq(evidence, Literature))
PHI-Base	Database of VFs involved in host-pathogen interactions	http://www.phi-base.org/

can be predicted using BLAST homology search against VFDB (Chen et al. 2016) or MvirDB (Zhou et al. 2007) (Table 3.2). However, the sequence homology-based identification of VGs can only predict the conserved VFs but is incapable of identifying potential VFs that are evolutionarily distantly related to known virulent proteins. To tackle this situation, several computational algorithms have been developed, such as VICMpred (Saha and Raghava 2006), to classify bacterial gene products for cellular processing, information, metabolism, and virulence. Two other sequence-based prediction methods Virulent-GO (Tsai et al. 2009) and VirulentPred (Garg and Gupta 2008) have been developed to predict bacterial virulent proteins. Network-based virulence factor prediction uses STRING database (https://string-db.org/) where protein-protein interaction networks give the information whether a given protein is a virulence factor or not (Zheng et al. 2012). VICMpred predicts the functions of gene products based on the sequence composition analysis and classifies them into cellular process, information molecules, metabolism, and virulence factors (Saha and Raghava 2006). To increase the accuracy of prediction method, a combination of two strategies, i.e., PSI-BLAST and Support Vector Machine (SVM), is used (Saha and Raghava 2006). The VF types identified by this method are toxins, adhesins, and hemolytic molecules. VirulentPred is an SVM-based method (Garg and Gupta 2008). This prediction method can predict the VFs from various potential protein sequences

including unknown and hypothetical protein sequences. Virulent-GO is another sequence-based method that uses instructive gene ontology terms as features for the identification of bacterial virulence proteins (Tsai et al. 2009).

3.3.4 Prediction of Antibiotic Resistance in Pathogenic Bacteria

The discovery of antibiotics was a boon to modern medicine and saved millions of lives. Usage of antibiotics is, however, a matter of great concern due to rapid emergence and spread of bacteria resistant to all classes of antibiotics. Consequently, resistance to multiple antibiotics of pathogens has become a complex healthcare issue threatening the medical management of infectious diseases. Resistance to antibiotics is a property which is sometimes natural or intrinsic to some bacteria and while at times arises by genetic mutations or acquisition via recombination from other bacteria (Bryant et al. 2012; Forsberg et al. 2012). The emergence of antibiotic resistance (AR) in bacteria is an ancient biological event, and antibiotic resistance genes existed in pre-antibiotic era as a defense mechanism against environmental microorganisms producing antibiotics (D'Costa et al. 2011; Crofts et al. 2017). Genomic studies of ancient DNA sequences have detected AR genes belonging to several classes, including β-lactams, glycopeptides, tetracyclines, and macrolides (D'Costa et al. 2011; Olaitan and Rolain 2016). The proliferation of AR in the era of modern medicine has been attributed to the rampant and inappropriate usage of antibiotics (D'Costa et al. 2011; Olaitan and Rolain 2016). Antibiotic resistance genes (ARGs) are sometimes either part of the chromosome or part of independent extrachromosomal elements, such as plasmids, bacteriophages, and integrative and conjugative elements (Ochman and Davalos 2006). Genes encoding resistance traits in microbes have not arisen spontaneously but have evolved from genes encoding housekeeping and metabolic proteins or small molecules produced in the environment by other organisms or geochemical reactions (Wright 2010). ARGs in environmental isolates are reservoirs of diverse potentially transferable resistance for pathogenic clinical isolates (Nesme et al. 2014; Crofts et al. 2017). The widespread recombination, including lateral or horizontal gene transfer from environmental resistome, leads to the emergence of multidrug resistance (MDR) among pathogenic bacteria (Forsberg et al. 2012; Perron et al. 2012). Therefore, understanding the consequences of evolutionary mutations (e.g., point mutations, insertions, deletions, or duplications) and bacterial recombination has important implications for controlling the spread of resistance pathogens and devising novel infection treatment strategies. The identification of ARGs has been facilitated by the availability of several public databases and bioinformatics tools, such as ARDB, CARD, ARG-ANNOT, ResFinder, etc. (Table 3.2). The Antibiotic Resistance Genes Database (ARDB) and Comprehensive Antibiotic Research Database (CARD) provide manually curated databases of ARGs and their products (Liu and Pop 2009; McArthur et al. 2013). Both ARDB and CARD facilitate the identification and characterization of novel ARGs in newly sequenced organisms using BLAST search program, and also provide information, such as resistance profile, type, and

mechanism (whether acquired resistance or mutational resistance) [Liu and Pop 2009; McArthur et al. 2013]. ResFinder is a web-based tool that uses BLAST search tool for identification of acquired ARGs as well as chromosomal mutations responsible for AR in bacterial genome sequence data (Zankari et al. 2012). ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) uses a local BLAST search program to detect existing and putative new ARGs in bacterial genomes (Gupta et al. 2014).

3.3.5 Horizontal Gene Transfers in Pathogen Evolution

Horizontal gene transfer (HGT) is the key driver of prokaryotic genome evolution by allowing the rapid acquisition of novel genetic elements encoding specific metabolic functions for adaptation to diverse ecological and host niche as well as virulence and antibiotic resistance (Wiedenbeck and Cohan 2011). However, the rates at which gene transfers occur are limited by the genetic and ecological similarity of organisms (Wiedenbeck and Cohan 2011). The main mechanisms of HGT are transformation, transduction, and conjugation. Transformation is a natural process and the primary mechanism by which some bacterial species take up small fragments of DNA from the environment, whereas the processes of conjugation and transduction are mediated by large mobile genetic elements (MGEs), which mediate their own transfer from one cell to another. MGEs carry large inserts of DNA that contain a number of functionally related genes putatively acquired by HGT and are often referred to as genomic islands (GIs) (Langille et al. 2010). Usually, GIs are identified by their atypical sequence composition and structure features, such as sporadic phylogenetic distribution, large size (> 8 Kb), abnormal G + C content, dinucleotide bias, distinct codon usage, high percentage of the mobility and phage-related genes, presence of neighboring tRNA genes, and flanking direct repeats, which discriminate them from the rest of the bacterial genome (Langille et al. 2010). Pathogenicity islands (PAIs) constitute a specific type of GIs that provide virulence properties to a wide range of Gram-positive and Gram-negative pathogens (Che et al. 2014a, b). The "mobility genes" of PAIs encode enzymes called integrases and transposases that catalyze their integration into the host chromosome. The exact mechanisms of PAI transfer from the donor to recipient organism are, however, not understood (Pallen and Wren 2007). A large number of phage-related genes are found in PAIs (Nakamura et al. 2004). PAIs contain more hypothetical (or unknown) protein-coding genes in comparison to the core genome (Hsiao et al. 2005) of pathogens. Insertion sequence (IS) elements are also very frequent in PAIs which can be predicted through searching of ISfinder database (Siguier et al. 2006). Some virulent genes also reside in PAIs, such as genes encoding type III and IV secretion systems, which form a specialized structure to recognize the host organism and assist to secrete effector proteins in host cell; invasins, which allow bacteria to invade epithelial cells; adhesins, which facilitate bacteria to adhere on host cells; toxins; and iron-scavenging system. The virulence factors are unequally distributed in genomic island; potent virulence factors (i.e., type III secretion system, type IV secretion system, and toxin) are often found in the pathogenic bacterial genomes

than in nonpathogenic ones (Che et al. 2014a). There are several bioinformatics tools and supporting databases (Table 3.3) available for prediction of GIs in prokaryote genomes, which follows the rule that closely related species preferably have similar genomic signatures. The Alien_Hunter (Vernikos and Parkhill 2006) is a sequence composition-based program which identifies genomic regions of "alien" origin that are present in various forms in the prokaryotic genomes. IslandPick, MobilomeFINDER, and MOSAIC are based on comparative genomic sequence analysis approach that can be used to find the PAIs (Ou et al. 2007; Chiapello et al. 2008). The advantage of comparative genomic-based PAI prediction is that it is easy to identify the dissimilarity among closely related genomes which are considered to have similar genomic signatures.

The other genomic elements with some of the signatures of GIs include integrative and conjugative elements (ICEs), bacteriophages, and plasmids (Hacker and Kaper 2000; Ochman and Davalos 2006). ICEs are the most widely distributed self-transmissible MGEs present in the genomes of diverse bacterial groups and play a significant role in genome plasticity, adaptation, and evolution (Johnson and Grossman 2015). They exhibit features of all other MGEs, such as phages, transposons, and plasmids, and encode regulatory systems to control their integration and excision from the chromosome, as well as replication and conjugative transfer (Johnson and Grossman 2015). The SXT/R391 family is the most well-studied ICE family. The SXT/R391 ICEs are among key players in the dissemination of heavy metals and MDR phenotype among environmental and pathogenic clinical isolates of the genera *Marinomonas*, *Vibrio*, *Shewanella*, *Photobacterium*, *Providencia*, and *Proteus* within the class *Gammaproteobacteria* (Badhai and Das 2016). All members of the SXT/R391family of ICEs share a conserved integrase that mediates site-specific integration into the 5′end of *prfC* or *tRNA*^{ser} in the absence of a *prfC* site and consists of 52 conserved core genes, some of which are involved in integration/excision, conjugative transfer, and regulation of the ICEs (Carraro and Burrus 2014). Other families of ICEs that are widespread in Gram-negative and Gram-positive bacteria and studied extensively to understand their biology and evolution are ICE*Bs*1 from *Bacillus subtilis*, CTnDOT from *Bacteroides thetaiotaomicron, ICESt1*/ICE*St*3 from *Streptococcus thermophilus*, ICE*clc* from *Pseudomonas putida*, ICE*Hin*1056 from *Haemophilus influenzae*, ICE*Lm*1 from *Listeria monocytogenes*, Tn916 from *Streptococcus aureus, and others* (Carraro and Burrus 2014, Johnson and Grossman 2015). Comprehensive genomic information of all reported ICE families is available in the ICEberg database (Bi et al. 2012), which has facilitated genomic comparisons and discovery of novel ICEs using BLAST (Altschul et al. 1990, 1997) or HMMER3 (Finn et al. 2011) search.

Bacteriophages are another class of MGEs that can mediate HGT. Genomic analyses of bacteriophages that have integrated into the bacterial chromosome (or prophages) revealed that it plays broad role in the diversification of pathogenic bacterial genomes. The main mechanism for horizontal gene transfer in prokaryotes is phage transduction, and it has been reported that 16% of *E. coli* O156:H7 strain Sakai genome is made-up of prophage (Canchaya et al. 2003). Prophages, derived from tailed bacteriophages, often carry excess genes within distinct "passenger

Table 3.3 Selected bioinformatics tools and databases for GI prediction

Software/tool	Application/description	URL
Alien_Hunter	Identification of horizontally acquired DNA	https://www.sanger.ac.uk/science/tools/alien-hunter/
GIST	Prediction of GIs in bacterial genome	http://www5.esu.edu/cpsc/bioinfo/software/GIST/
GIHunter	Detects GIs in both bacterial and archaeal genomes	http://www5.esu.edu/cpsc/bioinfo/software/GIHunter/
IGIPT	A web server used for genomic island prediction based on sequence composition analysis	http://bioinf.iiit.ac.in/IGIPT/
IslandPick	A computational tool for the detection of GIs	http://www.brinkman.mbb.sfu.ca/~mlangill/islandpick/
IslandPath	Identify genomic islands and potential pathogenicity islands	http://www.pathogenomics.sfu.ca/islandpath
SIGI-HMM	Detection of genomic islands and horizontal gene transfer, in prokaryotes	http://www.brinkman.mbb.sfu.ca/~mlangill/sigi-hmm/
IslandViewer	A computational method that incorporates three different GI prediction methods: IslandPick, IslandPath-DIMOB, and SIGI-HMM	http://www.pathogenomics.sfu.ca/islandviewer2/query.php
MobilomeFINDER	An online pipeline for identification of GIs based on tRNA gene locations and multiple genome alignments	http://db-mml.sjtu.edu.cn/MobilomeFINDER/
PredictBias	A web server that can predict PAIs based on their GC content, dinucleotide frequency, codon usage bias, and VF profile	http://www.bioinformatics.org/sachbinfo/predictbias.html
DGI	Database of genomic island contains GIs of >2000 bacterial genomes	http://www5.esu.edu/cpsc/bioinfo/dgi
Islander	A database for genomic island along with the information of tRNA and integrase genes	https://bioinformatics.sandia.gov/islander/
MOSAIC	A database that contains identical regions of bacterial genomes, identified based on comparative genomic analysis	http://genome.jouy.inra.fr/mosaic/
PAIDB	A database that contains pathogenicity island, potential pathogenicity island homologous to known pathogenicity island	http://www.paidb.re.kr/about_paidb.php
PlasmidFinder	An integrated web tool and database for identification of plasmids in completed or partially sequenced bacterial isolates based on curated database of plasmid replicons	https://cge.cbs.dtu.dk/services/PlasmidFinder/
pMLST	A web resource for typing of plasmids for identification of known plasmids, new alleles, and allelic variants	http://cge.cbs.dtu.dk/services/pMLST/

compartments" at one end of their genomes that often encodes virulence (Hendrix et al. 2000). Prediction of prophage gene can be done using HAMMER3 search against the Pfam protein database (Finn et al. 2011, 2014). Some classic VFs, such as diphtheria toxin, are encoded in the genomes of bacteriophages or prophages (Pallen and Wren 2007).

Plasmids are mostly circular DNA molecules that exist independently of the host bacterial genome. Plasmid genomes consist of a "core" backbone containing genes essential for its replication, segregation and conjugation functions, and a suite of "accessory" genes (Harrison and Brockhurst 2012). The "accessory" genes usually confer traits related to virulence; resistance to antibiotics, heavy metal, and toxic compounds; and various metabolic functions, such as nitrogen fixation, xenobiotics degradation, etc. that are advantageous to the host for niche-specific adaptations (Harrison and Brockhurst 2012). Often, the "accessory" genes present on smaller mobile elements allow their mobility within and between plasmids, as well as integration into the host chromosome (Harrison and Brockhurst 2012). Identification and typing of plasmids in both WGS-generated complete and draft bacterial genomes can be done using the web tools PlasmidFinder and pMLST (plasmid multilocus sequence typing) and the associated database (Carattoli et al. 2014).

3.3.6 Investigation of Disease Outbreak and Epidemiology

The precise identification of pathogenic strains is imperative for monitoring bacterial population dynamics, disease outbreaks, and epidemiological investigations. Most modern pathogens are thought to have evolved from ancestral free-living microbes residing in natural environments, such as soil and water, which serves as a mixing ground for the acquisition and dissemination of virulence and antimicrobial drug resistance traits that were earlier not present in specific groups of pathogens (Merhej et al. 2009). These acquired traits enable pathogenic bacteria to resist the increasingly difficult and hostile host immune defenses, as well as enable their host-to-host transmission during disease outbreaks. Conventional bacterial strain typing methods, such as genotyping based on single gene (16S rRNA or virulence gene) or repetitive elements, ribotyping, serotyping, biochemical typing, antibiotic resistance typing, etc., capture only a small subset of the variations among strains in a population and provide limited information for accurately distinguishing strains involved in an outbreak. Although these methods are useful in identifying pathogens in the short term for clinical diagnosis, their usefulness is severely limited in evolutionary studies (Bryant et al. 2012). Recombination and HGT events between bacterial genomes lead to acquisition of unlinked genomic regions with different evolutionary histories. Consequently, conventional microbial typing methods make identification of pathogen ambiguous and difficult. A portable modern molecular typing method called multilocus sequence typing (MLST) based on analysis of allelic profiles of individual genes or concatenation sequences of multiple housekeeping genes is being successfully used in unambiguous characterization of pathogenic strains (Maiden et al. 1998). The online MLST databases and resources

(Table 3.1) are providing critical support for epidemiological and evolutionary investigations. The expansion in the number of bacterial species for which large MLST datasets are available is aiding efforts to track the spread of emerging pathogenic strains and investigate their evolutionary dynamics. However, this molecular typing method does not provide any information beyond evolutionary relationship among strains. In this regard, NGS-based WGS has provided the best resolution in identifying strains involved in disease outbreaks, tracking sources or origins of the strains and their evolutionary relatedness (Gilchrist et al. 2015). Moreover, WGS coupled with computational biology tools has allowed comprehensive investigation of acquired gene content, identification of SNPs responsible for increased virulence and resistance phenotypes, as well as provide information on potential route of transmission during disease outbreaks (McGann et al. 2016).

3.4 Genomics Analysis in Understanding Bacterial Pathogenesis

3.4.1 Genomic Complexity and Plasticity of *Bordetella* spp. Causing Pertussis

Pertussis or whooping cough is an acute bacterial infection of the respiratory tract and a significant cause of morbidity and mortality in infants and children. Recent study has estimated >160,000 deaths due to pertussis in children below the age of 5 years (Yeung et al. 2017). It is a vaccine-preventable infectious disease; global vaccination efforts have led to significant reduction of morbidity and mortality (Wirsing von Koenig and Guiso 2017). However, the recent outbreaks of pertussis in countries with high vaccine coverage suggest that the disease is still not under control and remains one of the major threats to infants worldwide (Ausiello and Cassone 2014; Wirsing von Koenig and Guiso 2017). This resurgence in pertussis disease has prompted a relook into the existing vaccination strategy, as well as call for development of more effective vaccines to control transmission and prevent future outbreaks (Klein et al. 2017). The etiologic agent responsible for pertussis disease is *Bordetella pertussis*; however, to a lesser extent, pertussis-like symptoms are also caused by *B. parapertussis*$_{\mathrm{hu}}$ *and B. bronchiseptica* (Kilgore et al. 2016). *B. pertussis* is strictly a human pathogen; with no known animal or environmental. It is transmitted rapidly from person to person through contact with airborne droplets or aerosols (Mattoo and Cherry 2005; Cherry and Heininger 2014). *B. parapertussis* consists of two lineages; *B. parapertussis*$_{hu}$ exclusively infects humans and causes a milder form of pertussis-like disease in infants and pneumonia in adults, whereas *B. parapertussis*$_{ov}$ infects domestic animals, like sheep and swine, and causes chronic nonprogressive pneumonia (van der Zee et al. 1997; Cummings et al. 2004; Brinig et al. 2006). *B. parapertussis*$_{hu}$, like *B. pertussis*, lacks an environmental reservoir and is transmitted only via person-to-person aerosol route (Mattoo and Cherry, 2005; Cherry and Heininger, 2014). *B. bronchiseptica* causes infections ranging from lethal pneumonia to asymptomatic respiratory carriage and chronically

colonizes the respiratory tracts in a broad range of mammalian hosts, including horses, dogs, pigs, cats, and rabbits (Parkhill et al. 2003, Mattoo and Cherry 2005), with some lineages primarily isolated from humans (Diavatopoulos et al. 2005). Unlike, *B. pertussis* and *B. parapertussis*, *B. bronchiseptica* can survive in the natural environment and may be able to transmit via environmental reservoirs in addition to the usual host-to-host aerosol route (Akerley et al. 1992; Soumana et al. 2017).

Despite differences in critical aspects of pathogenesis, including host range specificity, disease transmissibility, severity, and persistence of infection, recent genomic studies have shown that the three classical *Bordetella* species are very closely related, sharing $\geq$98% nucleotide identity at the genome level and are referred to as subspecies (Parkhill et al. 2003; Diavatopoulos et al. 2005; Park et al. 2012). Multilocus sequence typing (MLST) has delineated strains of the three subspecies into four complexes: complex I includes *B. bronchiseptica* isolates preferentially of animal origin and the *B. parapertussis* ovine isolates (*B. parapertussis*$_{ov}$), complex II all *B. pertussis* isolates, complex III all *B. parapertussis* human isolates (*B. parapertussis*$_{hu}$), and complex IV *B. bronchiseptica* isolates preferentially of human origin. Phylogenetic studies based on MLST and whole genome sequences have suggested that the human pathogens *B. pertussis*, *B. parapertussis*$_{hu}$, and the sheep pathogen *B. parapertussis*$_{ov}$ have evolved independently, at least twice, from distinct ancestral lineages of the broad host range pathogen *B. bronchiseptica* (Parkhill et al. 2003; Diavatopoulos et al. 2005; Park et al. 2012). The strains of *B. pertussis* (complex II) were found to be more closely related to *B. bronchiseptica* (complex IV) than *B. parapertussis*$_{ov}$ (complex I) and *B. parapertussis*$_{ov}$ (complex III), suggesting their evolution from a human-adapted lineage of *B. bronchiseptica* ancestor, whereas the strains of *B. parapertussis*$_{ov}$ and *B. parapertussis*$_{hu}$ were closer to *B. bronchiseptica* (complex I) and may have evolved separately from an animal-adapted ancestral *B. bronchiseptica* lineage (Parkhill et al. 2003; Diavatopoulos et al. 2005; Park et al. 2012).

Although *B. pertussis* shares most of the genes of *B. parapertussis* and *B. bronchiseptica*, the genomes of *B. pertussis* isolates show a remarkable plasticity. Comparative genomics studies have revealed that the specialization of *B. pertussis* to a single host or a strictly host-restricted niche is accompanied by accumulation of pseudogenes, acquisition of a large number of insertion sequence (IS) elements, a massive reduction in genome size due to large-scale gene loss, and genomic rearrangements mediated by recombination between identical copies of the IS elements; whereas the broad host range of *B. bronchiseptica* has been attributed to the presence of a large repertoire of metabolic genes (Parkhill et al. 2003; Diavatopoulos et al. 2005; Park et al. 2012; Linz et al. 2016). The highest number of gene inactivation or pseudogenes formation by insertion of IS elements has been found in the genomes of *B. pertussis* followed by *B. parapertussis* as compared to the genomes of *B. bronchiseptica* (Parkhill et al. 2003; Diavatopoulos et al. 2005; Park et al. 2012; Linz et al. 2016). Despite the difference in genome size, the three classical *Bordetella* subspecies produce and secrete several common virulence

factors like toxins, adhesins, and other secreted proteins (Parkhill et al. 2003, Diavatopoulos et al. 2005, Park et al. 2012, Linz et al. 2016); the adhesins are required to attach to ciliated epithelial cells, macrophages, and neutrophils in the upper respiratory tract, while the toxins are required to survive host clearance systems (Mattoo and Cherry 2005, Abe et al. 2008). The common virulence factors include adhesins, such as filamentous hemagglutinin (FHA), autotransporter pertactin (PRN), and fimbriae proteins (FIMs); toxins, such as adenylate cyclase-hemolysin (ACT), dermonecrotic toxin (DNT), tracheal cytotoxin (TCT), and lipopolysaccharide endotoxin (LPS); and secretion system, such as a functional *bsc* type III secretion system (T3SS) [Parkhill et al. 2003, Melvin et al. 2014, Kilgore et al. 2016]. The main distinguishing feature of *B. pertussis* pathogenesis is the lymphocytosis caused by the production of pertussis toxin (PTX), which is lacking in *B. parapertussis* and *B. bronchiseptica*, due to mutation in the promoter region of the *ptx-ptl* locus encoding PTX and its secretion apparatus (Parkhill et al. 2003, Park et al. 2012). Another important feature of *B. pertussis* is the lack of expression of the O-antigen (lipopolysaccharide side chain) due to the insertion of an IS element in the O-antigen biosynthetic locus (Parkhill et al. 2003, Park et al. 2012). Other potential virulence factors such as outer membrane proteins OmpQ, tracheal colonization factor (TcfA), subtilisin-like autotransporters (SphB1), *Bordetella* intermediate-phase protein A (BipA), *Bordetella* colonization factor A (BcfA), pertactin homologous gene product (Phg), *Bordetella* autotransporter (BatB), and *Bordetella* autotransporter protein C (BapC) are species-specific and expressed by just one of the three subspecies (Melvin et al. 2014, Kilgore et al. 2016). The pertussis toxin (PTX) and serum-resistance factors (BrkAB) are expressed only by *B. pertussis* (Parkhill et al. 2003), whereas *B. bronchiseptica* is the only species producing a flagellum; five type III secreted proteins are BopC/BteA, BopB, BopD, BopN, BscN, and Bsp22 (Kurushima et al. 2012, Hegerle et al. 2013); and a type VI secretion system (T6SS) [Weyrich et al. 2012]. Genomic analysis has revealed that the loss of mobility in *B. pertussis* and *B. parapertussis* is due to disruption of the flagellar operon by the insertion of an IS element (Parkhill et al. 2003). The virulence activated gene *vag8* is expressed in *B. pertussis* and *B. bronchiseptica*, but not in *B. parapertussis* (Jacob-Dubuisson and Locht 2007). The three *Bordetella* subspecies may alter their phenotypic state depending upon environmental conditions and may show different expressions of virulence factors, which is critical for their pathogenicity (Cotter and Jones 2003). Genome-wide analyses have revealed that the coordinated regulation and expressions of these virulence factors and their associated export and/or modification systems are controlled by the *Bordetella* virulence gene (*bvg*) locus which encodes the BvgA/BvgS two-component regulatory system (Cummings et al. 2006, Nicholson 2007, Herrou et al. 2009, Moon et al. 2017). Moreover, comparative genomics data have revealed extensive polymorphisms in the virulence genes, particularly the five genes encoding the acellular pertussis vaccine antigens PTX, FHA, FIM2, FIM3, and PRN, as well as the pertussis toxin promoter. This could be the selection pressure of host-restricted evolution or due to vaccine-driven evolution within hosts (Cummings et al. 2004, Diavatopoulos et al. 2005, Park et al. 2012, Linz et al. 2016). Compared to most

other pathogenic bacteria, the genetic diversity in classical *Bordetella* subspecies is quite limited (Parkhill et al. 2003, Diavatopoulos et al. 2005). However, many recent publications have reported circulating strains of *B. pertussis* lacking expression as well as genes encoding major virulence factors, suggesting evolution and adaptation of *B. pertussis* is an ongoing process at a pace determined by the rate of accumulation of mutations and recombination (Bouchez and Guiso 2015,Williams et al. 2016). Several recent epidemiological studies have demonstrated that circulating *B. pertussis* strains differ from the vaccine strains; consequently, outbreaks of pertussis have occurred recently in countries with high acellular pertussis vaccine coverage (Ausiello and Cassone 2014, Bouchez and Guiso 2015,Yeung et al. 2017). These findings have prompted to relook into the current pertussis vaccination strategies and development of better vaccines, based on either whole cell or live bacteria, to control future pertussis disease outbreaks.

3.4.2 Genomics of Epidemic and Non-epidemic Strains of *Vibrio cholerae*

Vibrio cholerae belongs to *Vibrionaceae* family, is a facultative anaerobe Gram-negative bacterium, and causes severe diarrheal disease in human. *V. cholerae* spreads through contaminated water and food which leads to pandemic around the globe in 1961. *V. cholerae* is a multi-chromosomal bacterium and its genome is divided into two circular chromosomes (Heidelberg et al. 2000). Strains of *V. cholerae* are categorized into different serogroups on the basis of their O antigens, where O1 serogroup is mainly involved in epidemic and endemic cholera (Shimada et al. 1994). This serogroup is further divided into two biotypes, classical and El Tor, characterized by some biochemical properties or phenotypic markers (Reen and Boyd 2005). The cholera isolates that are non-agglutinable with the O1 antiserum are known as non-O1 serogroup of *V. cholerae* which are extensively found in aquatic environment and associated with sporadic diarrhea and extraintestinal infection (Shimada et al. 1994). Nevertheless, in 1992 *V. cholerae* non-O1 strain caused an outbreak of cholera epidemic throughout India and Bangladesh. *V. cholerae* 0139 (Bengal strain) that belongs to non-O1 serogroup of *V. cholerae* is designated as the main causative agent for the outbreak in 1992 (Faruque et al. 2003). A total of seven different cholera pandemics have been observed starting from the first pandemic in 1817. The etiological agent for seventh pandemic belongs to El Tor biotype of *V. cholerae* O1 serogroup that covers the highest geographic spread with the longest duration in comparison to all other pandemics (Pollitzer 1959). The availability of complete genome sequence of the seventh pandemic strain El Tor O1 N16961 facilitates to delineate the unknown fact about the evolution of pathogenic and environmental strains of *V. cholerae*.

Genomes of Pathogenic *V. cholerae* El Tor N16961 Complete genome sequence of *V. cholerae* El Tor N16961 strain has an estimated total 4033.46 kb size that encodes 3885 open reading frames (ORFs) (Heidelberg et al. 2000).

Realization of *V. cholerae* at the genome level is the beginning of understanding how the nonpathogenic bacteria transformed into a human pathogen. *V. cholerae* consists of diverse strains and biotypes which share toxin genes, antibiotic resistance genes, bacterial adhesion factor, and several other virulence factors. Various other species from *Vibrio* genus have been found to have pathogenicity for fishes and mammals. In the *V. cholerae* genome, most of the genes involved in cell growth, DNA duplication, transcription, translation, and major metabolic pathways are found in primary chromosome (large chromosome), whereas the genes for the intermediaries of metabolic pathways, ribosomal proteins, and hypothetical proteins are abundant in secondary chromosome (small chromosome) (Heidelberg et al. 2000). A large integron island in secondary chromosome contains 216 ORFs where most of the genes are involved in antibiotic resistance (resistance to chloramphenicol), DNA modification (transposes and integrase), and virulence (hemagglutinin). There is a marked difference in the gene distribution between the two chromosomes which are essential for virulence and growth of the bacterium. *V. cholerae* El Tor N16961 biotype strain caused the seven pandemics with severe diarrheal infection across the world and replaced the classical biotype (Heidelberg et al. 2000). There are three main components encoded by CTXφ which are required for a pathogenic *V. cholerae* strain such as cholera toxin, TCP pili, and *toxR* (Faruque et al. 1998). Comparison between the sixth pandemic classical strain and seventh pandemic El Tor strain shows a clear difference with regard to their colonization reaction, hemolytic activity, and antibiotic resistance. Microarray-based comparative genomic analysis gives insight about the genomic difference between these two pandemic strains and facilitates identification of the El Tor strain-specific unique genes (Dziejman et al. 2002). There are seven genes which are unique to El Tor strains encoding RTX toxin, hypothetical protein, and acetyltransferase located in the large chromosome and integron island in the small chromosome (Dziejman et al. 2002). The seventh pandemic El Tor strain-specific genes have also been characterized, and a total of 22 genes are thought to be obtained through horizontal gene transfer that consequently gave rise to *Vibrio* seventh pandemic island I or VSP I (Dziejman et al. 2002). Phylogenetic analysis of the sixth and seventh pandemic *V. cholerae* strains has revealed a big difference between genomes of pandemic strains due to the lateral gene transfer and acquisition of SNPs across the genome (Chun et al. 2009). The presence of VSP I and VSP II pathogenic islands in the El Tor strain makes it different from classical strain, and in addition, several other gene transfer events were also observed in the El Tor strain (Chun et al. 2009).

Pathogenicity Islands of *V. cholerae* Pathogenicity islands (PAIs) are pivotal for the evolution of pathogens, and it seems to play important roles to promote the mechanism of bacterial infection and the development of disease in the host body. Pathogenicity islands (PAIs) are clusters of unique genes in the genome of pathogens which contain putative virulence factors obtained through horizontal gene transfer and reside as a part of the genomic island. Pathogenesis mechanism of *V. cholerae* is complicated and requires several virulence factors to get in touch with the epithelial cells of the small intestine and make the enterotoxin to disturb the ion transport

channel of intestinal epithelium. Key virulence genes of *V. cholerae* are generally found as a cluster in both the chromosomes which include the CTX gene and TCP gene cluster, also known as TCP pathogenicity island. All the pathogenicity islands throughout the different species of pathogenic bacteria show similar gene content including the presence of transposes, integrase, recombination sites, and virulence genes. It is well established that TCP and cholera toxin are key responsible factors for human pathogenesis. In addition, horizontal transfer of CTX element and TCP pathogenic island is the possible mechanism for origin of new pathogenic *V. cholerae* strain. Antibiotic resistance patterns of different *V. cholerae* strains are changing with time, suggesting substantial acquisition of antibiotic resistance genes through horizontal gene transfer.

TCP Island Colonization at the intestinal epithelial cell is crucial for *V. cholerae* infection and is mediated by colonization factors encoded by the TCP gene cluster. TCP share the same chromosomal region together with ToxT encoding genes and other *acf* gene clusters. TcpA is the major subunit of TCP gene product that is encoded by *tcpA* gene in addition to a number of other genes located adjacent to the *tcpA* gene, leading to the formation of TCP gene cluster (Faruque et al. 2004). The entire region of TCP island is about 40 kb in size, and both ends are flanked by *att*-like sequence, and this island contains transposes, integrase, TCP, and *acf* gene clusters. A 13 kb upstream sequencing of TCP gene cluster reveals the presence of *tagA*, *aldA*, and four unknown genes; moreover, a marked homology for transposes and enteropathogenic *bfpM* genes was also observed (Faruque et al. 2004). *TCP* also acts as a receptor for CTXφ, so it is the major genetic element responsible for the evolution of epidemic *V. cholerae* strain.

CTX Island Multiple copies of *cholera toxin* (CT) genes are present in toxigenic *V. cholerae*. *Cholera toxin* is composed of two subunits named as A and B, encoded by two different overlapping ORFs. Another major toxin affecting the intracellular tight junction of intestinal epithelial cells to increase the permeability to bacteria is called as zonula occludens toxin (Zot). Zot gene is approximately 1.3 kb in size, present upstream to the *ctxA* gene encoding a polypeptide with 44.8 kDa molecular weight (Fasano et al. 1991). There is a third toxin in this island named as accessory cholera enterotoxin (Ace) responsible for fluid accumulation. Toxin-encoding genes like *ace*, *zot*, and *ctxAB* along with core-encoded pilin and unknown ORF are located on a core region that is flanked by multiple copies of the repetitive sequence; together, all these genetic elements constitute CTX pathogenic island (Fasano et al. 1991).The flanking repetitive sequences show divergence and consequently give information of two similar sequences referred to as RS1 and RS2 (Fasano et al. 1991).

VSP-1 and VSP-2 Island *Vibrio* seventh pandemic (VSP) island 1 and 2 are the cluster of genes grouped into two pathogenic islands responsible for the seventh pandemic caused by *V. cholerae* in 1961 (Faruque and Mekalanos 2003). These two islands emerged with the seventh pandemic strains of *V. cholerae* having low GC

content but absent from all the sixth pandemic strains of *V. cholerae*. The gene products of these islands are hypothetical protein essential for evolutionary fitness and to spread the seventh pandemic clone of *V. cholerae* (Faruque and Mekalanos 2003).

VPI 2 Island A 57 kb genetic element comprises genes involved in amino sugar metabolism and neuraminidase (nanH) found in VPI island (Karaolis et al. 1998).

Integron Islands *V. cholerae* genome also possesses a big cluster of genes known as integron island that acquires ORF and transforms it into functional genes. It is a 125.3 kb large gene cluster acting as an effective gene capture system. This island enables the bacterium to catch the genes from other microorganisms and thus develop a mechanism for clustering pathogenic genes and also the genes for other important molecular functions (Mazel et al. 1998). The role of integron island in the virulence of *V. cholerae* still needs to be explored and has important future prospects.

RTX (Repeats in Toxin) Gene Cluster It is a recently acquired gene cluster that plays an additional role in the virulence of *V. cholerae* producing exotoxins having leukotoxic and hemolytic activities like other pathogenic Gram-negative bacteria (Lin et al. 1999). The RTX toxin encoding gene cluster of *V. cholerae* is physically associated with the CTX prophage. Predicted ORFs in RTX gene cluster are named as *rtxA*, *rtxB*, *rtxC*, and *rtxD* (Lin et al. 1999).

Type IV-A Pilus Gene Cluster It is a 5.4 kb gene cluster similar to the tap gene cluster of *Aeromonas hydrophila* and operons of type IV-A pilus system. This gene cluster consists of five ORFs named as *pil*A, *pil*B, *pil*C, *pil*D, and *yac*E and is present in classical and El Tor both biotypes of *V. cholerae* strains where *pil*D and *pil*A are 100% conserved (Fullner and Mekalanos 1999). Several virulent proteins like prepilin peptidase, hemagglutinin, and TCP are encoded by this gene cluster (Fullner and Mekalanos 1999).

Regulation of Virulence Factor in *V. cholerae* Genome There are various systems that contribute to regulate the virulence-related genes in *V. cholerae*. All the putative virulent genes show coordinated expression due to the well-organized successive system of regulatory factors. A 32-kDa membrane protein ToxR playing a crucial role in the regulation of virulence that binds to the tandemly repeated DNA sequence upstream to the *ctxAB* gene enhances transcription of the *ctxAB*, resulting in high expression of cholera toxin (Champion et al. 1997; Faruque et al. 2004). Another transmembrane protein ToxS facilitated the activity of ToxR by assembling and stabilizing its monomers into dimers (Champion et al. 1997). Apart from *ctxAB*, ToxR also regulates 17 more genes such as genes for accessory colonization factor, OmpT, OmpU, TCP colonization factor, and so on (Champion et al. 1997). All the virulent genes regulated by ToxR are together known as ToxR regulon. ToxT is a 32-kDa protein that regulates the *toxT* gene

expression that activates other genes of the ToxR regulon so that ToxR becomes the main factor for the regulatory cascade involved in virulence of *V. cholerae* (Champion et al. 1997). Iron concentration is also associated with the regulation of virulent genes that encode several outer membrane proteins (OMP) and hemolysin. Low iron induces the expression of *fur* protein that binds to the promoter sequence repressing the expression of *viuA* and *irgA* genes (Goldberg et al. 1991). The *viuA* and *irgA* encode for outer membrane protein with molecular weight of 74 kDa and 77 kDa, respectively (Goldberg et al. 1991).

In Silico Prediction of Virulent Factor in *V. cholerae* Bioinformatics approach can be used for the prediction of a potential new virulence factor in *V. cholerae* genome. Protein interaction network and comparative genomics-dependent analysis of *V. cholerae* genome facilitated the prediction of candidate virulence-related genes present in the genome (Gu et al. 2009). Sequence analysis and homology modeling are emerging as major methods for identification of drug and vaccine target from the *V. cholerae* genome (Chawley et al. 2014).

3.4.3 Genomic Variations Among Contemporary *Salmonella enterica*

Salmonella genus, which belongs to *Enterobacteriaceae* family, is a Gram-negative, facultative anaerobic rod-shaped bacterium. The genus *Salmonella* diverges into two species *Salmonella enterica* and *Salmonella bongori* with more than 2500 serotypes (Tindall et al. 2005). There are a total six subspecies (subsp.) of *Salmonella enterica* which include subsp. I, subsp. II, subsp. IIIa, subsp. IIIb, subsp. IV, and subsp. VI characterized by biochemical tests and comparative genomics. Most of the serotypes of *S. enterica* species belonging to subsp. I cause both typhoidal and non-typhoidal salmonellosis (Tindall et al. 2005). The whole genome sequences of *S. typhimurium* LT2 (McClelland et al. 2001) and *S. typhi* CT18 (Parkhill et al. 2001) were annotated and published in 2001. These two genomes show significant synteny and collinearity with *E. coli* genome which confirmed that *Salmonella* is closely related to *E. coli. S. typhimurium* LT2 causes human gastroenteritis and is the main etiological agent for non-typhoidal salmonellosis leading to a large number of deaths each year (McClelland et al. 2001). Some serotypes are host specific like *S. typhi* (CT18) that infect only humans, whereas others like *S. typhimurium* are broad host range pathogens that exist in humans as well as in other mammals (Parkhill et al. 2001). The G + C content of *S. typhimurium LT2* genome is 53% that consists of 85 tRNAs, 7 rRNAs, and 11 structural RNAs (McClelland et al. 2001). *S. typhimurium* LT2 genome comprises a 4857 kb chromosome encoding 4489 CDS, and the 94 kb plasmid encodes for several virulence-associated genes (McClelland et al. 2001). Comparative genomics revealed that there are 204 pseudogenes in *S. typhimurium* and 39 in *S. typhimurium* LT2 chromosome (McClelland et al. 2001). The whole genome sequence of *S. typhi* CT18, the causative agent for typhoidal salmonellosis, has a 4858 kb chromosome and two plasmids of 218 kb and 108 kb, respectively

(Parkhill et al. 2001).The G + C content of *S. typhi* CT18 genome is 52.09% that consists of 78 tRNAs, 22 rRNAs, and 4599 coding sequences (CDS), while the plasmids contain 249 and 131 protein coding genes (Parkhill et al. 2001). *S. typhimurium* LT2 and *S. typhi* CT18 are well-characterized serotypes that show significant difference in their genomes and collection of virulence factor (Tindall et al. 2005). Different pathogenic islands, phages, and plasmids are responsible factors for the genetic diversity between the *S. typhimurium* LT2 and *S. typhi* CT18 strains (Sabbagh et al. 2010). Both the serotypes share approximately 89% of genes, while 600 genes are unique to *S. typhi* CT18, and 480 genes are unique to *S. typhimurium* LT2 (Parkhill et al. 2001). Genetic diversity of *S. typhi* CT18 genome is due to SNPs that cause formation of nonfunctional pseudogenes, whereas orthologs of many pseudogenes in *S. typhi* CT18 are fully functional in *S. typhimurium* LT2 (Sabbagh et al. 2010). Several prophage elements like Fels-1, Fels-2, Gifsy-1, and Gifsy-2 are interspersed throughout the *S. typhimurium* LT2 genome that confers virulence and leads to distinct genomic organization of this bacterium (McClelland et al. 2001).

***Salmonella* Pathogenicity Island (SPI)** Large number of virulence-associated genes of pathogenic *Salmonella* species are found in clusters known as *Salmonella* pathogenicity islands (Marcus et al. 2000). These virulent genes are common in many *Salmonella* serovar and are involved in cell invasion and intracellular pathogenesis (Marcus et al. 2000). Among the different types of SPI, some are unique for a single serovar, while others are conserved across the different serovars of *S. enterica* (Hensel 2004). SPIs are often located adjacent to the tRNA genes and have different G + C contents from the rest of the genome (Marcus et al. 2000). SPIs are horizontally transferred from plasmids, phages, or other unknown sources and have distinct sequence patterns from the other genes. A total of 11 common SPIs are found in the *S. typhimurium* and *S. typhi* genomes (Sabbagh et al. 2010). *S. typhimurium* has only one specific island, i.e., SPI-14, whereas *S. typhi* has multiple islands like SPIs-7, 15, 17, and 18 (Sabbagh et al. 2010).

SPI-1 SPI-1 is a 40 kb gene cluster with 42% of G + C content encoding type 3 secretion system that translocates effector protein molecules into the host cell. Type 3 secretion system facilitates bacteria to inject effector proteins into the host cell, resulting in cellular invasion and progression of infection. The genes encoding effector proteins can be located inside or outside of the SPI-1 (Sabbagh et al. 2010). The SPI-1 has complex regulation process; there are five regulators: HilA, HilC, HilD, InvF, and SprB. Except SprB, others contribute to regulatory pathways that consequence the activation of SPI-1 genes and of genes encoding type 3 secretion system (T3SS) effectors located outside SPI-1 (Marcus et al. 2000). Comparative study of SPI-I in *S. typhimurium* and *S. typhi* shows the presence of four additional ORFs including effector protein avrA and three cytoplasmic proteins STM2901, STM2902, and STM2903 in *S. typhimurium* genome (Sabbagh et al. 2010).

SPI-2 SPI-2 island is 40 kb sin size and consists of more than 40 genes located adjacent to the tRNA^{val} gene which encodes for second type 3 secretion system (T3SS) and a two-component regulatory system (Marcus et al. 2000). SPI-2 contains two distinct elements of 25 kb and 15 kb in size encoding T3SS proteins for cellular invasion and tetrathionate reductase (Ttr) for anaerobic respiration, respectively (Marcus et al. 2000). Comparison of this island does not reveal any major difference between *S. typhimurium* and *S. typhi* (Sabbagh et al. 2010). The regulatory mechanism of SPI-2 genes is simpler since it uses a two-component regulatory system encoded by *ssrAB* as the only transcriptional regulator for SPI-2 that activates genes encoding effector proteins of T3SS and the expression of SPI-2 genes (Deiwick et al. 1999). In addition to this, genes in the SPI-2 are crucial for prolonged bacterial growth in intestinal epithelial cells of the host body.

SPI-3 SPI-3 island is 17 kb in size, located adjacent to the tRNA^{selC} gene, and encodes for a magnesium transporter essential for intracellular survival of bacteria (Blanc-Potard et al. 1999). SPI-3 contains ten ORFs arranged in six transcriptional units including the *mgtCB* operon that encodes MgtC, a macrophage survival protein, and a $Mg2^{+}$ transporter protein MgtB (Blanc-Potard et al. 1999).The variation of this island between *S. typhimurium* and *S. typhi* found adjacent to the tRNA^{selC} gene has been characterized by large number of deletions and insertions (Sabbagh et al. 2010).

SPI-4 SPI-4 gene cluster is 24 kb in size and consists of six ORFs *sii* A, B, C, D, E, and F located next to the tRNA-like genes essential for type 1 secretion system and adhesin secretion for bacterial virulence (Wong et al. 1998). The G + C content of SPI-4 ranges from 37 to 44%, which is lower in comparison to the rest of the *S. typhimurium* genome (Wong et al. 1998). SPI-4 has 18 other putative ORFs involved in type I secretion system that facilitates toxin secretion similar to *E. coli* hemolysin secretion.

SPI-5 SPI-5 gene cluster is 7.6 kb in size with 43.6% GC content inserted next to the tRNA^{serT} locus and encodes effector proteins for T3SS encoded by both SPI-1 and SPI-2 (Wood et al. 1998). There is no difference between *S. typhi* and *S. typhimurium*, except STY1114, an additional ORF that encodes a transposase in *S. typhi* (Sabbagh et al. 2010).

***Salmonella* Chromosomal Island or SPI-6** SPI-6 island is 59 kb in size, located adjacent to the tRNA^{aspV} gene, and comprises the *saf gene* cluster that encodes an invasin and several products with unknown function (Folkesson et al. 2002). The entire deletion of this island in *S. typhimurium* reduces the invasion of *S. typhimurium* in cultured cell (Folkesson et al. 2002).

SPI-7 This is the largest pathogenic island in salmonella genome that is absent from *S. typhimurium* but present in *S. typhi* (McClelland et al. 2001). It is a 134 kb island inserted next to tRNA^{pheU} gene and contains nearly 150 ORFs that encode

several virulence factors like SopE effector, Vi antigen, and type IVB pili (Pickard et al. 2003).

SPI-8 It is 6.8 kb in size, located near tRNA^{pheV} gene encoding putative virulence factor bacteriocins, and it is widely distributed among salmonella serotypes (Hensel 2004).

SPI-9 This gene cluster is 16 kb in size present in both the serotypes (*S. typhimurium and S. typhi*) and inserted next to the lysogenic bacteriophage (Sabbagh et al. 2010). This island contains several genes that encode virulence factors such as type I secretion system and an RTX-like protein (McClelland et al. 2001).

SPI-10 SPI-10 is a 32.8 kb island located adjacent to the tRNA^{leuX} gene, and it contains a cryptic bacteriophage (McClelland et al. 2001). The function of this bacteriophage in the distribution of SPI-X has not been elucidated so far. The major virulence factor encoded by this island is Sef fimbriae (Townsend et al. 2001).

SPI-11 SPI-11 was identified following the genome sequencing of *S. choleraesuis* (Chiu et al., 2005). This is a 14 kb island located adjacent to the *Gifsy*-1 prophage. Many genes on this island have been identified for their role in virulence of *S. typhimurium*. The *pagC* and *pagD* genes are regulated by *slyA* and the *phoP/phoQ* two-component system where pagC contributes to intracellular survival and systemic infection in mice (Gunn et al. 1995). The *envE* and *envF* genes are predicted to encode envelope proteins but not required for virulence in mice (Gunn et al. 1995).

SPI-12 It is 15.8-kb- and 6.3-kb-long island present in *S. typhimurium* and *S. typhi*, respectively, inserted next to the tRNA^{proL} gene (Hansen-Wester and Hensel 2002). The single virulence factor in SPI-12 is SspH2 which secretes effector protein of T2SS that influences the rate of actin polymerization inside the infected cells (Hansen-Wester and Hensel 2002).

SPI-13 SPI-13 is a 25 kb region located next to the tRNA^{pheV} gene in *S. typhimurium* and in *S. typhi* (Haneda et al. 2009). Among the 18 genes in this island, only 3 contribute to virulence, viz., *gacD*, *gtrA*, and *gtrB* (Haneda et al. 2009).

SPI-14 SPI-14 is a 9 kb island that contains seven ORFs encoding cytoplasmic proteins (Shah et al. 2005). Two genes *gpiA* and *gpiB* are required for the virulence of *S. gallinarum* in 1-day-old chicks; however, functions of other genes in this island are unknown (Shah et al. 2005).

SPI-15, 17, and 18 (*S. typhi*-specific PAI) All these three islands are present in *S. typhi* but absent in *S. typhimurium*. The SPI-15 gene cluster is 6.5 kb in size containing five ORFs that encode hypothetical proteins and is located near the

tRNA^{glyU} gene in *S. typhi* (Vernikos and Parkhill 2006). SPI-17 is 5 kb in size and comprises six ORFs located next to tRNA^{argW} site (Vernikos and Parkhill 2006). SPI-18 is a 2.3 kb sequence and contains only two ORFs encoding virulence factors ClyA and TaiA. ClyA, a pore-forming cytolysin, facilitates invasion of human epithelial cells, while TaiA, a secreted invasin, enhances bacterial uptake by human macrophages (Fuentes et al. 2008).

Regulation of Virulence Factors of *Salmonella* The virulence genes of *S.* serover are clustered in a specialized region of *Salmonella* chromosome known as *Salmonella* pathogenicity islands (SPI). Acquisition of unique virulence genes of SPI-2 is a major factor for the divergence of *S. enterica* and *S. bongori* to separate species (Fookes et al. 2011). There are three putative regulatory systems that contribute to the virulence of *Salmonella*: OmpR/EnvZ, PhoP/PhoQ, SsrB/SsrA, and OmpR/EnvZ. The PhoP/PhoQ regulates SsrB/SsrA system of SPI-2 where SsrA is a histidine kinase and SsrB is a response regulator (Miller et al. 1989). The SsrB/SsrA system is composed of histidine sensor kinase (SsrA) and response transcription regulator (SsrB). The PhoP activates the SsrB that involves in type three secretion system of *Salmonella* serovar. The OmpR/EnvZ system facilitates the direct binding of OmpR response factor to the promoter that induces the expression of ssrAB and confirms synergistic role of both PhoP/PhoQ and OmpR/EnvZ systems in the regulation of *Salmonella* virulence (Garmendia et al. 2003).

3.5 Conclusions

Microbes, particularly bacteria, play an indispensable role in human health and various diseases. Unrecognized pathogens in the environment, domesticated animals as well as hospital settings can easily cause disease outbreaks and put human lives at great risk. Thus, identification and characterization of both emerging and well-recognized pathogenic microorganisms are imperative for successful disease prevention, treatment, and control. Advances in NGS technologies and computational biology tools have allowed comprehensive investigation of the gene content, structure, and likely function of microbial genomes, particularly useful in identification of intrinsic, as well as acquired virulence and antimicrobial resistance properties of pathogenic microbes. Genomic analyses have provided greater insight to understand the evolution, virulence, and pathogenesis of infection caused by the three classical *Bordetella* species. The strictly human-adapted *B. pertussis* and *B. parapertussis*$_{\mathrm{hu}}$ and the ovine-adapted *B. parapertussis*$_{\mathrm{ov}}$ have evolved from the different broad host ranges *B. bronchiseptica* or *B. bronchiseptica*-like ancestor. Despite lack of genetic diversity, the differences in their virulence, disease severity, persistence, and host specificity have been attributed to extensive gene polymorphism, acquisition IS elements, gene inactivation, genome decay, and genomic rearrangements. Moreover, genomic data have suggested the evolution and adaption of *B. pertussis* is an ongoing process; consequently, epidemic outbreaks are common phenomena even in countries with high vaccine coverage. Advancement of genomic techniques has

made it possible to characterize pathogenic island (PAI) by studying the dynamics and complexity of the pathogenic genome. Study of *V. cholerae* genomes gives insight about the location of horizontally transferred virulence genes (PAIs) in its genome and helps to identify them. The analysis of pathogenic islands of *V. cholerae* and *S. enterica* using the genomic technique unfolds the responsible genetic element and its role for the development of pathogenesis. Comparative genomics and sequence analysis have opened the way to identify gene clusters in pathogenic *Vibrio* and *Salmonella* species, which consequently clear our understanding of their mechanism of pathogenesis and their course of evolution. Understanding of virulence determinants in PAIs using genomics analyses has made it easy to investigate the potential drug or vaccine targets for bacterial infections.

3.6 Future Prospects

The implementation of novel statistical phylogenetic algorithms and NGS technologies-based sequencing methodologies will give us insights into the evolutionary pressures on bacterial pathogens to facilitate development of predictive models on future emergence and spread of clones with enhanced virulence and antibiotic resistance phenotypes.

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4 Fungal Diversity: Global Perspective and Ecosystem Dynamics

Apekcha Bajpai, Seema Rawat, and Bhavdish N. Johri

Abstract

All major biomes on earth contain a multitude of microorganisms; of this, a considerable proportion is shared by fungi in terms of abundance, genetic diversity, biomass and total biospheric DNA. In various ecosystems, fungi exist as pathogens, mutualists and decomposers and are of considerable ecological value as they influence nearly every component of the ecosystem services, viz. protection against pathogens, homeostatic balance, decomposition and other functions. Fungi are, however, functionally redundant in some ecosystems and endemic to certain bioregions. Next-generation sequencing has now uncovered unculturable fungal forms that has transformed our understanding towards their role in unexplored environments; cataloguing their diversity and study of their biogeographical patterns at local and global scale have become simpler. The data generated through advanced molecular approaches have introduced the concept of 'mycobiome' which was largely overlooked or considered as an integral yet small component of the 'microbiome' until now. In this chapter, we report new information that reveals various deterministic factors that shape fungal communities and their probable role in maintaining human, soil and plant health. Finally, we also discuss how the view of mycobiome has taken an independent shape and has more recently helped understand interkingdom interactions.

Keywords

Fungi · Mycobiome · Endemic · Diversity · Microbiome

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4.1 Diversity Perspectives

Microbial diversity is highly heterogeneous and comprises bacteria, cyanobacteria, archaea, fungi, protozoa and viruses. In addition, enumeration of microbial species—considering it as an ecological priority—has commenced back in 1988, indicating microbes to be 5% of all species on the planet (May 1988). However, robust sequencing technologies have expanded these estimates for microbial species inhabiting the planet earth to 1 trillion (Locey and Lennon 2016). In this time course, dramatic changes in the number of microbial species were reported in various studies which are reflected in the number of representatives present in individual kingdoms. A recent report from Royal Botanic Gardens, Kew (https://stateoftheworldsfungi.org/2018/reports/SOTWFungi_2018_Full_Report.pdf), has detailed the status of fungal world encompassing both fundamental and applied aspects. According to this compilation, 350 new families within the fungal kingdom have been described during the last decade. The largest among these include *Mycosphaerellaceae* comprising 6400 species, *Pucciniaceae* with 5000 species and *Agaricaceae* and *Cortinariaceae* consisting of 3000 species. During the year 2017 alone, 2189 new fungal species have been recorded; of these, genus *Inocybe* is represented by 179 species collected from Australia, Europe and India. In the new additions, 68% comprised ascomycetous forms, whereas *Basidiomycetes* consist of 30% of fungi described. A new addition represented by *Dominikia emiratia* from desert soils appears to be involved in the sustenance of plants at elevated temperatures. Two new lichenicolous additions include *Talpapellis mahensis* and *Strictographa dirinariicola*. Based on this detailed report of fungal world, it can be concluded that fungi are much more diverse than previously envisaged. Use of molecular tools is going to play an important role in future estimates of global fungal diversity. Some of these issues are discussed in the following sections of this chapter.

4.1.1 Number Game: From Hawksworth to Hawksworth!

Fungi originated around 1 billion years ago and are one of the most diverse eukaryotic kingdoms encompassing 144,000 species that are named to date (https://stateoftheworldsfungi.org/2018/reports/SOTWFungi_2018_Full_Report.pdf). As evident from various studies, fungi are inevitably present everywhere, and attempts to enumerate their numbers and taxonomic diversity abound in literature. An early attempt by Hawksworth (1991) estimated fungal diversity conservatively at 1.5 million species; subsequently, 1.5 million could be considered as a working hypothesis (Hawksworth 2001). Further studies have suggested an altered ratio estimating fungal forms to be 3.5–5.1 million species (O'Brien et al. 2005; Blackwell 2011). Some of these uncertainties are a result of hidden fungal forms in plant world which is largely influenced by geographical location and edaphic factors. More recently, Tedersoo et al. (2014) conducted a study of 365 sampling sites globally and stated that the fungal diversity is overestimated by 1.5–2.5 times.

Therefore, this varying ratio has led Hawksworth and Lucking (2017) to revisit the actual diversity of fungi to be within the range of 2.2–3.8 million species.

4.1.2 Single Cell vs. Hyphal Forms

Fungi are found in free-living, filamentous or symbiotic forms (Treseder and Lenon 2015). Morphologically they range from single-celled forms like yeast to several kilometre-long filamentous organisms such as *Armillaria solidipes* (Ferguson et al. 2003). Yeasts have better stress tolerance than filamentous forms (Schimel and Bennett 2004). Filamentous forms have added advantage over yeast forms in terms of longer hyphae that help in rapid decomposition, nutrient translocation and carbon mineralization (Li and Fahey 2013). Being present in such large numbers, they have profound impact on various earth biomes through decomposing diverse polymers and synthesizing biological compounds in dead or live host and biomolecules used as foods for humans.

4.1.3 Biospheric DNA vs. Fungal DNA

Based on mass distribution across various biomes of earth, the biosphere contains 5.3×10^{31} Mb of DNA incorporated in 2×10^{12} tonnes of biomass. Such study has expanded our knowledge of different kingdoms of life in terms of DNA equivalence; prokaryotes (1.6×10^{31}); unicellular eukaryotes (1.3×10^{31} Mb); animals (4.2×10^{29}); plants (3.6×10^{31}); viruses (4.0×10^{29}); fungi thus contribute 1.7×10^{27} Mb of DNA less than plant or animal matter. Uncertainties in DNA content in the biosphere arise due to lack of large-scale data on biomass densities for fungal groups (Landenmark et al. 2015). However, consistent efforts by scientists have now recently quoted 12 gigatons of carbon biomass encompassed by fungi on the planet earth (Bar-on et al. 2018); such studies are likely to contribute towards better quantitative analyses of biospheric components.

4.1.4 Endemicity vs. Diversity

What invokes curiosity to study fungi includes their cosmopolitan distribution as also their occurrence in extremophilic habitats including polar regions, deserts and deep oceans while surviving extreme temperature in cold seeps (−1.8 °C to 2 °C) and deep-sea vents. Bacteria are far more predominant in extreme temperatures although some lithotrophic and heterotrophic forms of fungi have recently been explored in environments such as subglacial ecosystem, polar regions, deserts and deep oceans. Regardless of the fact that a few trees are harboured in boreal region, they have been found to be colonized by diverse fungal species; in a study of one such area encompassing a radius of ~150 m, a large number of basidiomycetous forms have been reported in Lunsen forest, Sweden (Fig. 4.1). Leaves of tropical trees are considered to be hotspots of fungal diversity (Arnold and Lutzoni 2007).

Fig. 4.1 Fungal diversity at high-latitude boreal forests showing the diverse range of fruiting bodies including those of ectomycorrhizal, saprotrophic and pathogenic fungi. (Wardle and Lindahl 2014, reprinted with permission)

Table 4.1 An estimate of fungi in different biomes

S. no.	Biome	Number	References
1.	Planet earth	2.2–3.8 million	Hawksworth and Lucking (2017)
2.	Marine environment	<1000	Grosberg et al. (2012)
3.	Terrestrial environment (global soil)	*Basidiomycota* (55.7%), *Ascomycota* (31.3%), *Mortierellomycotina* (6.3%) and *Mucoromycotina* (4.4%)	Tedersoo et al. (2014)
4.	Human	>390 species	Gouba and Drancourt (2015)
	Gut	>50 genera (dominant are *Candida*, *Saccharomyces* and *Cladosporium* sp.)	Hoffmann et al. (2013)
	Oral	>75 genera (dominant are *Candida*, *Cladosporium*, *Aureobasidium*, *Aspergillus* and *Fusarium*)	Ghannoum et al. (2010)
	Foot	>40 genera (dominant are *Malassezia, Aspergillus, Cryptococcus, Rhodotorula and Epicoccum*)	Findley et al. (2013)
	Skin	*Malassezia, Penicillium. Aspergillus, Candida, Rhodotorula, Cladosporium* and *Mucor*	Findley et al. (2013)

Supporting this hypothesis, Schimann et al. (2017) reported greatest diversity of fungi in tropics due to the presence of high vegetation content, litter availability, prevailing humidity and temperature. Metagenomic investigations have further helped in studying some extreme environments such as subglacial ecosystem (Doyle et al. 2018), deep-sea vents (Tisthammer et al. 2016), polar regions (Cox et al. 2016), deep biosphere igneous rocks (Drake and Ivarsson 2017) and atmosphere (Woo et al. 2018); this has further included early colonization of human system beginning with child birth (Koenig et al. 2011; Heisel et al. 2015) (Table 4.1). Lichenologists are exploring environments like Neotropical lowland forests and have surprisingly found basidiolichens in such areas; *Sulzbacheromyces* and *Dictzonema* are not confined to dry biomes, whereas a new species *Cora itabaina* has been reported from lowland area (Cáceres et al. 2018). Symbiotic fungal association with plants termed as mycorrhiza is a very well-established system and is thought to be several-million-years-old association; nearly 50,000 fungal species are associated with 2,50,000 plant species (van der Heijden et al. 2015). Among these, ectomycorrhizal species comprise 7950 taxa distributed across 234 genera (Comandini et al. 2012), ericoid diversity consists of about 150 taxa (Walker et al. 2011) and orchidaceous mycorrhizae comprise ~25,000 taxa (van der Heijden et al. 2015). Two classes, *Dothideomycetes* and *Agaricomycetes*, are abundant in atmospheric fungal assemblages (Woo et al. 2018), and deep-sea vents are dominated by the genus *Malassezia* (Amend 2014). However, being highly diverse, some fungal groups are reported to be endemic, owing largely to their dispersal limitation. Soils are dominated by *Ascomycota* (31.3%) and *Basidiomycota* (55.7%), and they are

endemic to tropical regions (Tedersoo et al. 2014); geographical endemism in soil fungi is attributed to dispersal limitation in the group. At local scale, fungal communities are controlled by climate and host plant identity, whereas dispersal constraints them at global scale (Talbot et al. 2014).

4.1.5 Culturable vs. Unculturable Forms

In 1988, May had proposed that fungi and plants comprise about 22% of the existing species diversity. However, use of metagenomics and other tools in studying fungal diversity has now revolutionized the scenario, and the estimates have drastically changed (Zoll et al. 2016); an exact number of 1,20,000 known species belong to this kingdom (Hawksworth and Lucking 2017). A significant proportion of unculturable fungi have now been recovered by targeting ITS region; microsatellites or SNPs have been used to determine the genetic diversity (Araujo 2014). According to these investigations, unculturable fungal species vary from 101 to 207 (Gouba and Drancourt 2015; Mukherjee et al. 2014).

A comparative study conducted on 161 participants, comprising healthy and different stages of hepatitis B patients, provides stark contrast in the use of culturable and unculturable tools. Culture-dependent studies revealed the presence of *Saccharomyces* and *Candida*, whereas 377 different OTUs belonging to *Saccharomyces, Penicillium, Galactomyces* and *Cryptococcus* spp. were recorded through unculturable tools (Chen et al. 2011). Hence, next-generation sequencing has increased the depth of identification of species which is probably not attainable by culture-based methods.

4.2 Diversity and Habitat Relationships

4.2.1 Freshwater vs. Marine Habitat

Fungal taxonomic diversity in marine ecosystem is relatively dominated by *Ascomycetes* and *Basidiomycetes* in both deep-sea water and shallow-sea water, while other groups are relatively absent at a depth of 4000 m (Bass et al. 2007; Tisthammer et al. 2016). Yeasts are commonly found in this region, including some filamentous forms representing four fungal branches at a depth of 4000 m (Tisthammer et al. 2016), whereas freshwater streams are rich in *Hyphomycetes* (Krauss et al. 2011). Fungi are not only found in free-living form but are also present in symbiosis with marine invertebrates and sponges, which are excellent sources of novel secondary metabolites. Predominant among these are the genera *Acremonium, Alternaria, Aspergillus, Cochliobolus, Curvularia, Fusarium, Humicola* and *Penicillium*. Fungal species in marine ecosystem are quite similar to terrestrial forms; relatively few species which are divergent are probably adapted to deep-sea habitat by altering their membrane composition under high hydrostatic pressure (Simonato et al. 2006). Factors such as oxygen, depth and nitrate affect fungal diversity than

geographical distance; significantly distinct communities in Pelagic and Benthic zones have therefore been separated (Baas et al. 2007). This suggests that marine fungi might follow biogeographic patterns similar to terrestrial microbes at global scale.

Marine fungi have excellent potential in industry and climate change scenario, especially the filamentous forms recovered from sea grass *Posidonia oceanica*, the green alga *Flabellia petiolate* and the brown alga *Padina pavonica*; these are good source of hydrophobins which show emulsification property and stable amphiphilic films (Cicatiello et al. 2016). Additionally, they are a rich source of novel bioactive compounds that possess antibacterial, anti-inflammatory, antiplasmodial, anticancer and antiviral properties (Blunt et al. 2016). Metagenomics of bacterial sequences in Global Ocean Sampling Expedition reveals the presence of dddP gene involved in conversion of dimethylsulfoniopropionate (DMSP) to dimethyl sulphide (DMS); surprisingly, certain species of ascomycetous fungi *Aspergillus oryzae* and *Fusarium graminearum* have also to been reported harbour this gene. DMS is important in regulating the climate by increasing the formation of cloud droplets that in turn reduce the amount of sunlight reaching the oceanic surface (Todd et al. 2009).

4.2.2 Endospheric vs. Phyllospheric Habitat

The understanding of distribution of fungal community in aboveground and below-ground habitats is essential, as they together play a vital role in biogeochemical cycling (Averill et al. 2014) along with contributing to the plant health (Busby et al. 2016). The analysis of functional guilds would help in understanding the fungal ecology in local as well as global scale. The plant roots are considered to be a 'well-delimited ecological compartment' in which endosphere constitutes a 'restricted area' (Vandenkoornhuyse et al. 2015). The microbiota occurring in endosphere was considered as contaminant for a long time, it is well known that they have an intimate relationship with the plant (Mitter et al. 2013). The cultivation-independent approaches and fluorescence in situ hybridization-confocal laser scanning microscopy (FISH-CLSM) have been useful in assessing the endophytes.

The presence of more complex organic molecules as nutrient source in the endosphere may play a role in determining the structure of microbial communities residing there. Endosphere provides a more protected environment as compared to other habitats as it is not exposed to environmental fluctuations. Fungi are considered to be important component of endosphere for plant nutrition and resistance to all kinds of stresses (Le van et al. 2017). Moreover, the endophytes can respond rapidly to the alterations in the physiology of plants in response to changing environmental conditions. The composition of fungal communities residing in endosphere will be significantly different from the rhizosphere as well as soil communities (Schlaeppi et al. 2014; Fonesca-Garcia et al. 2016). However, the assembly rules underlying coexistence of a number of fungal taxa in endosphere is poorly understood.

The dispersal limitation, interspecific competition and environmental filtering can be the mechanisms for phylogenetic clustering of fungi in endosphere (Helmus et al. 2007). The 'kin-selection' strategy may also contribute in structuring the

composition of fungal community (Le Van et al. 2017). Le Van et al. (2017) determined the taxonomic composition of endospheric fungal community of 19 co-occurring *Agrostis stolonifera* plants. It is considered to be the first study which demonstrated that the 'phylogenetic signal of the fungal root microbiome was phylum dependent'. Thus, the endosphere fungal communities are not randomly assembled but are 'specifically filtered by their plant host'. The phylogenetic structure exhibited a large number of clustering at host-plant scale than expected by null model. Yang et al. (2016) also reported that the endophytic fungi of *Betula ermanii* were more phylogenetically clustered as compared to soil fungi. Thus, host plants can select their fungal partners. However, Gang et al. (2017) reported that the community structure of endosphere fungi of 'first-class endangered wild plant *Cypripedium japonicum*' in Korea was similar to that of rhizosphere fungi, though former exhibited more diversity.

Phyllosphere, representing the plant-air interface, has an estimated global leaf area of 109 km^2 (Woodward and Lomas 2004). It is a complex habitat, characterized by rapidly changing abiotic conditions. It provides important substrate for fungal growth. Fungal communities, viz. saprophytic, pathogenic and epiphytes, though present on leaf surface are not as abundant as bacterial community. It is still unknown as to what are the major drivers of fungal community in phyllosphere (Vorholt 2012). The physical and chemical characteristics of leaf surface govern the growth of fungi as it provides a permanent physical environment suitable for the growth and reproduction of saprophytic fungi, while for phytopathogens, it provides a temporary surface (Allen et al. 1991). Bodenhausen et al. (2014) and Ritpitakphong et al. (2016) reported that besides the physical and chemical adversities, secondary metabolites produced by plant, antimicrobial wax layers, trichomes and hairs determine the structure of phyllosphere communities.

Yang et al. (2016) reported that the phyllospheric fungi of *Betula ermanii* were more phylogenetically clustered as compared to soil fungi, suggesting the filtering action of host plants. Sapkota et al. (2017) studied the phyllosphere mycobiome using metabarcoding of fungal ITS1 region. They took samples from three uppermost leaves at three growth stages from four cultivars grown at two different locations of Denmark to understand the effect of spatiotemporal variation upon mycobiome. They observed that though geographical location had a major effect upon the structure of mycobiome, leaf position, cultivar and growth stage exhibited significant effect upon the mycobiome. The variations in older leaves were attributed to the variation in cultivars, while the variation in younger leaves was due to variation in geographical location. The rapidly growing fungi were found to occupy the empty space in leaf surface. They observed negative coexistence between *Zymoseptoria tritici* and yeasts Cystofilobasidiacea, *Dioszegia* and *Sporobolomyces*.

The growth of non-pathogenic fungi depends upon the nutrients deposited from atmosphere on leaf surface or those exuded from leaf and environmental conditions like temperature, water availability and UV radiation (Belanger and Avis 2002; Sundin 2002; Levetin and Dorsey 2006). The atmosphere contains a large quantity of fungal spores which may adhere to the epidermis (Andrews and Buck 2002). The release of fungal spores from phylloplane is usually passive mediated by wind or rain

splash; however, in some cases, they may be actively propelled (Kinkel 1997; Aylor 2002; Levetin 2002). Levetin and Dorsey (2006) reported that the source of many fungi that colonize the leaf surface is air spores. The spores deposited on leaf surface can germinate or may re-entrain into the atmosphere.

4.2.2.1 Mycorrhizal Relationship and Ecological Adaptation

The plant-fungi symbiotic relationship is approximately 400 million years old and is found in approximately 90% of all land plants from forest trees to grasses to crops. The fungus either produces a sheath around the plant root, which then produces hyphae that grows into the root and out into the soil (Ectomycorrhizae) or the hyphae grow within the cells and out into the soil (Endomycorrhizae). The mycorrhizal fungi form network between the nearby plants via hyphal connections (Smith and Read 2008). The functional role of mycorrhizal network in the physiology of host plants and functioning of ecosystem is still debatable. It is proved that the mycelium absorbs soil nutrients and supplies them to host plant and thus plays a major role in nutrient cycling though its role in carbon flux is ill-defined and also controversial (Selosse and Roy 2009). However, it has been reported that the achlorophyllous plants in forest understory use the mycorrhizal fungi associated with nearby autotrophic trees as the carbon and energy sources (Roy et al. 2009).

Endomycorrhizal species of *Ascomycetes* and *Basidiomycetes* are said to have shaped the forest structure as they interact with trees of Caesalpinoidaceae, Dipterocarpaceae, Fagaceae and Pinaceae which are the dominant trees of many forests. The omics helped in unravelling the mechanism and effector molecules involved in formation of symbiotic association, uptake and exchange of nutrients between the symbiotic partners (Bonfante and Genre 2010). The comparison of genomes of ectomycorrhizal fungi *Laccaria bicolor* (Martin et al. 2008) and *Tuber melanosporum* (Riccioni et al. 2008) revealed the primary factors involved in regulating the development and function of mycorrhiza (Bonfante and Genre 2010). It has been observed that only 1.5% of protein-coding genes are differentially regulated during symbiotic-association formation (Riccioni et al. 2008). The presence of a huge number of transposable elements representing more than 20% of genome in *L. bicolor* (Martin et al. 2008) and 58% of genome in *Tuber melanosporum* (Riccioni et al. 2008) suggests their involvement in genome evolution and plasticity (Bonfante and Genre 2010). The gene-encoding enzymes targeting plant cell wall and others involved in avoiding the defence reactions of plants are upregulated during the symbiotic process (Bonfante and Genre 2010).

The nutritional strategies are found to be different in symbiotic fungi as indicated by their ability to access glucose, sucrose, fructose etc. The genes encoding hexose transporters and catabolic enzymes are upregulated during symbiosis. The mechanism of exchange of nutrients like nitrogen etc. between mycelium and plants is not well understood. Glutamine and ammonium have been observed to cross the symbiotic interface. This indicates that the exchange takes place either by the assimilation of ammonium into amino acids in mycelium and their translocation into the plants or direct release of ammonium ions from Hartig net hyphae. The upregulation of

ammonium importers in Endomycorrhiza poplar roots suggests the direct release of ammonium from Hartig net hyphae (Bonfante and Genre 2010).

The mode of communication between fungus and the host plants vary. The upregulation of cysteine-rich secreted proteins like MISSP7 in the hyphal mantle of *L. bicolor* suggests that either they function as effectors during early phase of symbiotic interaction or are involved in the construction of symbiotic interface (Martin et al. 2008). However, the absence of these effector molecules in other EM-like *T. melanosporum* indicates that there are other alternative pathways involved. The investigation of 39,303 genes on poplar microarray revealed the differential expression of 2945 genes, including the genes encoding proteins involved in root morphogenesis and metabolism of auxin.

Arbuscular mycorrhizal (AM) fungi belong to the *Glomeromycota* phylum and are the most widespread fungal symbionts. These contribute significantly to increasing the plant biomass and improving the resistance of plants to stress and pathogens and also in uptake of plant nutrients. AM host plants can survive in absence of fungi, while AM fungi cannot be cultured in the absence of host. A high degree of genetic and functional variability is observed within this group (Bonfante and Genre 2010). The investigation of unculturable endobacteria within *Glomeromycota* suggested that they are the ancient partners of AM fungi and are also of evolutionary significance in fungal speciation (Naumann et al. 2010).

The expression of effector proteins helped in understanding the functions of fungi. The localized expression of high affinity P_i transporter in the extraradical hyphae of *G. versiforme* suggested that the polyphosphate is rapidly translocated along the aseptate mycelium to the host plants. The preferential expression of glutamine synthase gene in extraradical hyphae and gene associated with arginine breakdown in intraradical mycelium of *G. intraradices* suggested their role in transport of ammonium and amino acids. Till date, no hexose transporters have been characterized in *Glomeromycota* (Bonfante and Genre 2010).

The mechanism of root colonization by AM fungi has been conserved during evolution. Root colonization is essential for AM fungi. Strigolactones and other bioactive molecules are being released by plant roots which are sensed by the presymbiotic mycelium and thus help fungi identify their host plants. These bioactive molecules stimulate the branching and metabolism of fungus. The fungi reciprocate to this signal by secreting diffusible signals called 'Myc factor' which are partially lipophilic molecules and smaller than 3 kDa (Kosuta et al. 2003). These signals are perceived by plants via Myc factor receptor which in turn induces the expression of a membrane-bound steroid-binding protein, considered to be important for mycorrhization (Kuhn et al. 2010). These factors also play a critical role in communication between nitrogen-fixing bacteria and AM fungi. The plants have a common signal transduction pathway, called symbiosis (SYM) pathway, responsible for association with AM fungi and Rhizobia. The binding of Myc factor on receptor induces a rapid transient elevation of secondary messenger, calcium ions (Ca^{2+}). The calcium ion sensor 'Cameleon' expressed in root hairs of *M. truncatula* sense the Ca^{2+} oscillations induced in the perinuclear cytoplasm due to the proximity of branched hyphae (Bonfante and Genre 2010).

Markmann et al. (2008) reported that in *Lotus japonicus*, *LjSYMRK* (*MtDM12* in *Medicago truncata*) codes for a kinase receptor which perceive the signals of *Rhizobium* and AM fungi. All downstream elements of SYM pathway are found to be localized in nucleus which suggests the rapid transduction of signal to nucleus. The two nucleoporins (*LjNUP85* and *LjNUP133*) are also found in this pathway. *LjCASTOR/LjPOLLUX* (*MtDM12* in *Medicago truncata*)-encoded cation channels, located in the nuclear envelope, are found to be involved in calcium spiking (Parniske 2008). The onset of repeated Ca^{2+} oscillations in nucleus and perinuclear cytoplasm through the alternate activity of Ca^{2+} channels and transporters require *MtDM1* and *MtDM12* (Oldroyd and Downie 2004; Bonfante and Genre 2010). *LjCCaMK* (*MtDM13*)-encoded calcium-calmodulin-dependent protein kinase is found responsible for decoding calcium oscillations. This kinase phosphorylates the product of LjCYCLOPS (last SYM gene), leading to colonization (Yano et al. 2008).

The chemical acquaintance established between the fungus and plant results in proliferation and branching of roots and hyphae. This presymbiotic phase ends when the hyphal tip touches the surface of the root, i.e. establishment of physical contact between the two partners. The fungal hyphae then wander along the root surface. They form straight or gently curved hyphae which then swell on the cell wall of root epidermal cells and undergo repeated branching to develop hyphopodium which adhere to the root epidermis. The molecular mechanism that mediates this development is still not clearly known. However, the expression of genes change, and expression of genes involved in cell wall remodelling and defence become active. The fungus growth is halted for 4–6 h after the development of hyphopodium, and during this period, plant cells prepare for colonization by fungus. The epidermal cells reorganize to produce prepenetration apparatus (PPA), AM-specific structure, and these contacted epidermal cells start to assemble the secretory machinery involved in building of the interface compartment where the fungus will reside. At the contact site, cytoplasm becomes aggregated, and thereafter, a thick column is developed which predicts the route of penetration of hypha (Genre et al. 2005). All elements, viz. endoplasmic reticulum, Golgi bodies and secretory vesicles, are concentrated in PPA. The movement of nucleus to and from the contact site is the critical factor in PPA development (Genre et al. 2005). Thereafter, growth of new tip is initiated from which the penetration hyphae is developed which invade the plant tissues. The repeated branching of intracellular hypha results in the formation of arbuscules in the inner root cortex which are the sites of nutrient exchange (Paszowski 2006). The genes involved in this process include *Vapyrin* gene (codes for a protein of unknown function), but its inhibition results in a significant decrease in the penetration of epidermis and blocking of arbuscule formation (Pumplin et al. 2010), subtilisin (Takeda et al. 2009), phosphate transporter (Javot et al. 2007) and AABC transporters (Zhang et al. 2010). The differential expression of more than 500 genes, like those encoding for transcription factors, nutrient transporters and proteins involved in the synthesis of cell wall and cellular dynamics, has been reported during the arbuscular phase in a number of plants like *M. truncata* and *L. japonicus*. It has been observed that the inhibition of expression of sucrose synthase gene reduces the plant height, shoot weight, seed yield and arbuscule development (Baier et al. 2010).

4.2.3 Diseased vs. Healthy Human Habitat

Unlike human microbiome, the fungal component 'mycobiome' has been poorly characterized in healthy and diseased individuals. About 390 fungal species in 50 genera are reported commensally on human body; of these, *Saccharomyces, Candida* and *Malassezia* are dominant in gut of healthy subjects which is affected by diet, age and gender (Hoffmann et al. 2013). However, in diseased condition, certain alternations occur in the fungal community composition (Gouba and Drancourt 2015) which is well exemplified by the following cases. In a comparative study of obese and non-obese individuals, *Mucor racemosus* and *Mucor fuscus* were predominant in the latter, suggesting altered mycobiota (Rodriguez et al. 2015). Greater prevalence of *Candida* has been reported from patients exhibiting autoimmune diseases like type 1 diabetes (Gosiewski et al. 2014) and autism spectrum disorders (Strati et al. 2017). Also in case of HIV-infected patients, high prevalence of pathogenic forms of *Candida*, *Aspergillus* and *Fusarium* genera was detected in the oral cavity (Mukherjee et al. 2014). Such studies support the notion that most of the mycobiome composition is altered under diseased condition. Studies on mycobiome took the centre stage when Iliev et al. (2012) showed the role of fungi in IBD. Dectin 1 receptors present on epithelial cells recognize fungal cells and Dectin 2, 3 recognize α-mannon found on fungal hyphae. It has been found that mice deficient in Dectin 3 are more susceptible to chemically induced colitis than wild-type mice. (Underhill and Iliev 2014). An increase in the proportion of pathogenic *Candida* and *Trichosporon* and a decrease in the non-pathogenic *Saccharomyces* were noted in mice during colitis. Role of gut bacteria in maintaining neurological-intestinal and extraintestinal balance is well demonstrated (Mu et al. 2016); however, gut mycobiome axis has recently emerged as an important component (Enaud et al. 2018). Mycobiome dysbiosis is associated with IBD, and its role is linked to gut-brain axis as it is characterized by altered cognitive functions, hypothalamic-pituitary-adrenal axis (HPAA) dysfunctions with lower total cortisol levels (Jeffery et al. 2012). Evident from several studies, there occurs a fungal dysbiosis in psychiatric and genetic disorders as well as schizophrenia which is characterized by an expansion of *Candida* and *S. cerevisiae* (Severance et al. 2016). High abundance of *Candida* is reported in Rett syndrome, a genetic neurological disorder (Strati et al. 2017. *Lactobacillus rhamnosus* and *Bifidobacterium animalis* significantly reduced blood levels of *C. albicans* antibodies and improved psychiatric symptoms (Foster et al. 2017).

4.2.4 Arctic vs. Antarctic Forms

Latitude is an important deterministic factor in structuring microbial communities. Taxonomic diversity of bacteria is known to peak at mid-latitude whereas fungi are reported to decrease poleward (Bahram et al. 2018); a nearly similar pattern is followed by endophytic fungal species as their diversity increases from arctic to tropical sites (Arnold and Lutzoni 2007). However, this trend reverses in case of

ectomycorrhizal fungi which show lower diversity in tropics (Tedersoo and Nara 2010) owing to the poor soil profile, nutrient resource availability and lack of plant species. In contrast, total fungal biomass and the fungal/bacterial biomass ratio increases poleward, owing to their greater tolerance to nutrients and long-distance transport of water through hyphae (Bahram et al. 2018). A study by Treseder et al. (2014) has shown that beyond plants and animals, principle of 'tropical conservatism hypothesis' could be applied to microbes as well which relates to successful linking of evolutionary history and biogeographic distribution of fungi at large scale. As a consequence of this consideration, exclusive presence of phyla such as *Entomophthoromycota*, *Mucoromycotina*, *Zygomycota*, *Glomeromycota*, *Ascomycota* and *Basidiomycota* preferably occupies higher latitudes, lower temperatures and low-precipitation states. This could be attributed to the fact that they spend much of their life in an enclosed protective cell wall throughout their life cycle and lack zoospore stage unlike older phyla which exhibit zoospore stage and are vulnerable to drought.

Using high-throughput sequencing, Cox et al. (2016) showed that Antarctica shares significantly more fungi compared with the Arctic and displays lower endemism as also strong bipolar distribution due to similar environmental conditions prevailing in the polar region. Predominant fungi that show bipolar distribution belong to the classes *Leotiomycetes*, *Eurotiomycetes*, *Sordariomycetes*, *Agaricomycetes*, *Microbotryomycetes* and *Tremellomycetes*. Endemism in Antarctic fungi largely belongs to the classes *Eurotiomycetes* and *Dothideomycetes*. Fungi in these groups show competitive advantage over presence of other fungal groups through development of tolerance to extremes of temperature, drought, UV radiation and by production of rock-mineralizing enzymes (Treseder and Lennon 2015). Recently, two contrasting ecozones representing mushroom adaptability were reviewed by Halbwachs and Simmel (2018). According to these authors, fungi under such extreme environments are exposed to permafrost, frost churning and repeated freeze-thaw cycle conditions that are harmful to fungal mycelium. To circumvent such extreme situations, fungi rescue themselves by accumulating melanin, trehalose and mannitol in their cell wall and extracellular matrix (Onofri et al. 2007); induction of chaperones occurs under thermal stress (Tiwari et al. 2015), and alcohols, sugars and unsaturated lipids act as antifreeze compounds in the membrane to maintain low water content (Moser 1993).

4.3 Ecosystem Dynamics

4.3.1 Degradation of Plant Matter and Carbon Turnover

An understanding of the diversity and abundance of microbial species in an ecosystem is currently an ecological priority due to their contribution in nutrient dynamics and recycling of various elements (Bar-on et al. 2018); making sense of their numbers has therefore been a frontier to main aim of research for mycologists world over. Fungi are the third major component constituting around <10% of the

global biomass after terrestrial plants and bacteria; fungi provide excellent ecosystem services that sometimes outplay those exhibited by bacteria in terms of productivity (Hassan 2017). Besides, they are also important candidates in governing biogeochemical cycles, plant nutrition and pathology. Anaerobic fungi in deep crust, a little known ecosystem, are significant hydrogen gas provider which is utilized by bacteria to drive autotrophic processes (Drake and Ivarsson 2017). Apart from marine bacteria, a few members of *Ascomycetes* are also now known to utilize dimethyl sulphide that is involved in regulating global sulphur cycle and climate (Todd et al. 2009). Fungi are chiefly involved in decomposition of cellulose and lignin (Tresedor and Lennon 2015), releasing simpler compounds into the environments which are in turn consumed by small invertebrates which find complex molecules difficult to digest. Fungi are a good source of food for both animals and humans as they depend partially or wholly on them, e.g. caribuo, potoroo and banana slugs depend wholly on lichens or fungi over other foods. Both wild-type mushrooms and mushrooms which are difficult to cultivate such as truffles, matsutake and morel are consumed as food by humans; *Amanita phalloides,* however, is highly poisonous and has been termed as death cap mushroom. Beside consumption as food, they are also utilized as an excellent source of antibiotics like penicillin, vitamins (thiamine and riboflavin) and in industrial production of Bakers' yeast (Kendrick 2001).

4.3.2 Contribution in Climate Change

4.3.2.1 Fungal Spores in Cloud Formation

The annual emission of spores, hyphae and propagules of fungi from pedosphere into atmosphere is estimated to be 28–50 Tg (Elbert et al. 2007; Heald and Spracklen 2009). These emitted fungal particles form cloud-condensation nuclei (CCN) and ice-forming nuclei (IN) (Pöschl et al. 2010; Hassett et al. 2015) besides absorbing and reflecting solar radiations (Guyon et al. 2004). The fungal spores along with other biological aerosol particles provide the surface area upon which the condensation and crystallization water drops and ice crystals contributing to creation of cloud, fog and precipitation (Fröhlich-Nowoisky et al. 2009). The particle size affects the flux densities of fungal deposition. The aerodynamic diameter of fungal particles is taxon dependent (Yamamoto et al. 2012). It has been estimated that particle size with diameter greater than 2 mm acts as an effective CCN which can collide efficiently and coalesce with smaller droplets contributing to the cloud precipitation (Möhler et al. 2007). The knowledge regarding fungal taxa in atmosphere is very scarce. The culture-based approaches are able to identify only 10–40% atmospheric fungi, of which *Fusarium* species is the most commonly encountered. *Basidiomycetes* were rarely detected by cultivation approach; however, basidiospores have been shown to form effective cloud nuclei (Hassett et al. 2015).

Mushroom spores are considered to be powerful catalyst for raindrop formation in clouds; 30,000 basidiospores can be released every second by a gilled mushroom which corresponds to a billion of microscopic particles (Money 2011).

Basidiospores are released by a catapult mechanism. The secretion of mannitol and other hygroscopic sugars stimulates the condensation of water drop on the surface of spore resulting in the formation of Buller's drop which merges with the adaxial drop on adjacent spore surface and thereby causes a rapid displacement of the centre of mass of the spore (Webster et al. 1989, 1995). The surface tension drives the fluid motion which imparts momentum to the spore resulting in the launch of spores at the initial velocity of 1.8 m/s (Stolze-Rybczynski et al. 2009). The spores are dispersed in airflow around the mushroom cap. Similar mechanism of spore discharge has been reported in poroid mushrooms (Hassett et al. 2015). The discharge of a large number of basidiospores into the atmosphere above forests results in the condensation of water in clouds.

Woo et al. (2018) reported that large multicellular spore-producing *Dothideomycetes* (da $\geq$ 10.0 μm) were predominant in dry deposition, as revealed by high-throughput sequencing and quantitative PCR. They observed higher taxonomic richness of fungal community in wet deposition. *Dothideomycetes* and *Agaricomycetes* were found to be the predominant fungi in air. *Perenniporia* and *Trametes* were found to be the two most abundant *Agaricomycetes*, while *Alternaria, Cladosporium* and *Epicoccum* were observed to be the three most abundant *Dothideomycetes*. The fungal assemblage members and structures were found to be different in dry and wet depositions. The environmental perturbations change the patterns of precipitation which is mediated by the change in the fungal communities along with other microbial communities in the atmosphere.

The different roles of basidiospores in the dynamics of cloud formation have been proposed. Haga et al. (2014) proposed that the fungal spores may either operate as ice-nucleating agents at low temperatures. Hassett et al. (2015) proposed that hygroscopic basidiospores, being large aerosol particles with diameter exceeding 2 μm as observed by scanning electron microscope, operate as giant cloud-condensation nuclei (GCCN) which coalesce with smaller droplets resulting into the formation of larger precipitation-sized drops (Mohler et al. 2007). The formation of raindrop occurs by the coalescence of water droplets with GCCN rather by the condensation of water vapour (Cotton and Yuter 2009). Non-ballistosporic basidiospores may also act as nuclei, but they act in the same manner as non-biological origin particulates.

4.3.2.2 Regulation of Biogeochemical Cycles

Fungi also play an important role in cycling of elements in the biosphere. However, their role has been frequently neglected. The aeromycological processes like sedimentation and precipitation contribute to global biogeochemical cycles. The symbiotic mycorrhizal fungi are the key players in all the major transformations and cycling of nutrients, whereas free-living fungi are the important candidates for the decomposition of organic materials. In freshwater ecosystems, fungi are instrumental in the transfer of energy from riparian forest to aquatic ecosystems by decomposing wood and leaf litter that falls into the water, while in terrestrial ecosystems, fungi transfer the energy from above the ground to below it, where it is recycled back to the plants.

The bulk of organic matter inputs into streams is contributed by riparian vegetation. It contributes up to 99% of organic carbon in woodland stream (Webster and Meyer 1997). The leaf resources enter as pulse in temperate streams which is rapidly colonized by aquatic fungi along with bacteria. The hyphae of aquatic *Hyphomycetes* secrete an array of hydrolytic enzymes which digest the polysaccharides of leaf resulting into the assimilation of organic carbon by fungi and release of particulate organic carbon in streams. The fungal biomass contributes to 18–23% of total mass of detritus which is consumed by detritivores (Methvin and Suberkropp 2003). Consequently, fungi occupy the central position in the trophic structure of food webs which mediate the carbon cycling and flow of energy to higher trophic level. The studies on subtropical and temperate freshwater wetland indicated that fungi participate in the 'aerial standing-dead litter phase' which can contribute to carbon and nutrient cycling (Gulis et al. 2006).

Nitrification and denitrification are the important processes of global nitrogen cycle. A wide range of fungi are capable of nitrification in terrestrial ecosystems (Falih and wainwright 1995). Shoun et al. (1992) reported that fungi are also capable of denitrification. The fungi have also been reported to dominate both microbial nitrification and denitrification in soil (Laughlin and Stevens 2002). Shoun et al. (1992) reported that aquatic fungi are capable of nitrification and denitrification. Newell (1993) reported that fungi could account for substantial portion of total plant litter nitrogen. Suberkropp (1995) reported that aquatic fungi can utilize nitrogen from both organic substrates as well as overlying water. Most aquatic *Hyphomycetes* can use both organic and inorganic nitrogen, while some chytrids and oomycetes are unable to utilize nitrate (Gulis et al. 2006). A significant contribution of fungi to total detrital nitrogen has been reported in freshwater wetlands (Findlay et al. 2002).

Mycorrhiza also play critical role in regulation of biogeochemical cycles. AM fungi provide effective pathway to scavenge phosphorus from large soil volume and delivery it rapidly to root cortical cells. This phosphorus transport flux is fuelled by carbon flux in the opposite direction (Jansa et al. 2010). The hyphal network of AM fungi absorbs inorganic phosphorus via high affinity P_i transporters which is translocated rapidly to the plant roots. The active uptake by plants is facilitated by the H^+-ATPases which energize the plasma membrane of plants surrounding the intracellular structures of fungi (Smith and Read 2008). They can mediate processes which can bypass the mineralization of organic phosphorus by free-living decomposers by secreting a number of hydrolytic enzymes and organic acids which in turn release the recalcitrant organic and mineral phosphorus in the soil (Jansa et al. 2010). The ecto- and ericoid-mycorrhizae have been well known to influence nitrogen-cycling process. Bender et al. (2015) reported that AM fungi can enhance the sustainability of N and P cycling. AM fungi were found to reduce the leaching of P by 31%, enhance plant phosphorus by 15% and increase P mobilization in soil by 18% in experimental grassland microcosms. The leaching of nitrogen was reduced by 24% in healthy soil, whereas plant nitrogen was increased by 13% in pasture soil but not in healthy soil.

4.3.3 Contribution to Human Health

Fungi are normally found in all body sites and are generally assumed to be associated with diseases. However, culture-independent studies have uncovered commensal species associated with human body (Heisel et al. 2015); culture-dependent techniques have unearthed them in low abundance from such habitats (Palmer et al. 2007). Human gut colonization by fungi starts early in life; infant gut colonization by *C. albicans*, *Leptosphaerulina* and *C. parapsilosis* (Koenig et al. 2011) has now been studied in considerable detail (Huseyin et al. 2017). In commensal state, these fungal forms can modulate human immune response and can work as immune boosters.

Another aspect of fungal community studies in human ecosystem includes perturbation in fungal composition upon dysbiosis which can be used as a diagnostic tool to understand the aetiology of diseases. The gut mycobiome is now linked to diseases such as irritating bowel syndrome (IBD), hepatitis B and several others. Available data show that human body is host to distinct fungal genera and perturbations may result in altered community through factors such as gender (Strati et al. 2017), age (Heisel et al. 2015), diet composition and others (Hoffman et al. 2013).

4.3.3.1 Use as Probiotics

Live microbial feed supplements which confer beneficial effects on host by improving their intestinal microbial balance are termed as 'probiotics' (Fuller 1989). In human system, fungi are in commensal state but can cause disease when host defence or competitive exclusion becomes compromised, e.g. *Cryptococcus* is found in 20% of oral samples of healthy individuals (Ghannoum et al. 2010); however, under dysbiosis state, it causes cryptococcosis. Therefore, influence of mycobiota and dietary fungi in maintaining homeostasis in mammals suggests a pharmacotherapeutic potential of modulating the fungal composition which could be treated with the use of probiotics and faecal transplantations. *Saccharomyces cerevisiae* var. *boulardii* is known for its probiotic effect that results on account of interference with metabolic activities and immune system (Rima et al. 2012). Oral administration of this yeast has been successfully utilized in treatment of gastrointestinal diseases such as IBD (Didari et al. 2015). Moreover, improvement in gastrointestinal neuromuscular anomalies was also observed upon probiotic treatment (Botschuijver et al. 2017). It has been suggested that the probiotic action of *S. boulardii* includes modulation in host cell-signalling pathways through decrease in the expression of cytokines such as IL6, IL8, IL1β and TNFα and TNFγ (Mumy et al. 2007) as also by neutralization of bacterial toxins. In addition, new evidence links the increased production of IgA, modulation of immune response, inhibition to inflammatory response and proinflammatory cytokines like TNFα and IL-6 in *S. cerevisiae* and *C. albicans* as well; latter is also associated with blockage of NO production (Enaud et al. 2018). However, presence of specific microsatellite allele, trisomy of chromosome 9 and altered copy number of genes influencing enhanced growth rate and better survival strategies makes *S. boulardii* different from *S. cerevisiae* (Zanello et al. 2009).

Apart from *Saccharomyces*, other yeast strains like *Issatchenkia occidentalis*, *Kluyveromyces marxianus* and *Kluyveromyces lactis* have been found to perform better when used as a probiotic in terms of growth and survival rates; they can withstand stress conditions such as pH, elevated concentration of bile salts, pepsin and production of protease (Moradi et al. 2018). Oral administration of *Candida kefyr* has been shown to protect mice from autoimmune encephalomyelitis (Takata et al. 2015). Instead of using whole organisms, current research is directed towards application of fungi-derived molecules as a substitute for antibacterial agents and preservatives in food industry (Mogilnicka and Ufnal 2018).

4.3.4 Use as a Diagnostic Marker

Available reports show a distinct shift in fungal composition in individuals diagnosed with IBD, hepatitis B and some psychiatric disorders (Trojanowska et al. 2010; Sokol et al. 2017). McKenzie et al. (1990) have shown a distinct relationship between *C. albicans* and Crohn's disease. Strains of *C. albicans* recovered from the oral cavity were found to be similar in their genotypic state to those isolated from different segments of gastrointestinal tract, suggesting fungal transmission from oral cavity to gastrointestinal tract. There was also a shift in the profile of *Candida* species; healthy individuals showed the presence of *C. tropicalis* whereas patients suffering from IBD were colonized with *C. glabrata, C. krusei, C. guilliermondii, C. kefyr, C. tropicalis and Geotrichum candidum* (Trojanowska et al. 2010). In addition, predominance of five fungal genera, viz. *Saccharomyces, Clavispora*, *Cyberlindnera*, *Candida* and *Kluyveromyces,* was observed in Crohn's disease (Lewis et al. 2015). A recent study by Sokol et al. (2017) reports an increase in *Basidiomycota-Ascomycota* ratio, together with increased proportion of *C. albicans* and *Malassezia sympodialis* with concomitant decrease in *S. cerevisiae* in IBD patients compared with healthy volunteers.

Apart from gastrointestinal disorders, diseases such as Kawasaki, which affect coronary artery, show positive correlation with fungal antigens (Ishibashi et al. 2014). On a similar front, fungi such as *Tetratrichomonas* sp., *Aspergillus ruber, Penicillium solitum* and *Cladosporium bruhnei* have been found in anorexic patients but not in human gut (Gouba et al. 2014). Therefore, altered community of fungi in the oral cavity can be utilized as diagnostic marker in detection of such diseases.

4.4 From Microbiome to Mycobiome

4.4.1 Soil Mycobiome

Domain bacteria and fungi are ubiquitously distributed in nature; however, bacteria predominate in abundance and in terms of biospheric DNA (Landenmark et al. 2015). Therefore, fungi were always included in the study of microbiome as a rather smaller component. This scenario has changed since Ghannoum et al. (2010) used

the term mycobiome to represent this underrepresented component of the microbiome. Subsequently, extensive studies have been taken up to signify the role of fungi in various biomes. It is now fairly well established that fungi constitute a major portion of biomass on terrestrial biome as compared to bacteria in marine biome although primary productivity of the two environments is estimated to be roughly equal (Bar-on et al. 2018). Mycobiome-related investigations face many challenges, including their known level of lower abundance and recovery of fungal DNA which may be more difficult (Huffnagle and Noverr 2013). Mycobiome is found to be less persistent than microbiome, and the concept of core mycobiome has not received a firmer push from mycologists. However, Nash et al. (2017) provided a possibility that a core mycobiome may exist after analysing gut mycobiome of humans dominated by *Saccharomyces, Malassezia* and *Candida*. This notion gets strength from the observation that fungi found at various body sites are more specific than their bacterial counterparts (Findley et al. 2013). Recently, it has also been found that in indoor environments, fungi seem to be less readily transferred (Tong et al. 2017) than bacteria (Wilkins et al. 2016). These features make mycobiome a separate entity of the biome that has multitude of ecological functions.

Fungi in soil are important representatives of terrestrial ecosystem. *Ascomycetes* and *Basidiomycetes* constitute 98% of all known fungal species in soil (James et al. 2006). Globally, efforts are currently underway to produce a gene catalogue (http://www.earthmicrobiome.org/) from soil samples to discern the total microbiome across the world. As evident from these studies, *Ascomycota* and *Basidiomycota* are the dominant phyla in soil samples, but plant colonization alters the overall community of soil. *Zygomycota* members have been found as frequent colonizer of rhizosphere of *Crocus sativus*, interestingly implying that fungal diversity is niche and growth stage specific (Ambardar et al. 2016). Considering plants fungal communities in phyllosphere milieu, mycobiome make up is affected by plant genotype and is a major factor in shaping fungal distribution than does the geographical distance (Cordier et al. 2012a, b).

Sun et al. (2017) showed remarkable differences between fungal and bacterial communities and concluded that majority of the change in the former occurred over longer time period than those for bacteria; it has been observed that community shift in soil bacteria changes, whereas fungal communities exhibit a distinct profile over a time scale. As a result of this and other investigations, it can be inferred that resilience and biogeographic patterning in fungal communities is variable. Tedersoo et al. (2014) provide a deep insight into the global ecology of soil fungi; they further show a distinct relationship wherein increase in the ratio of fungal to plant diversity rises with latitude. In addition to latitude, soil fungal community is strongly influenced by distance from the equator and with mean annual precipitation (Talbot et al. 2014). Human activities have also been found to influence the role of soil fungal communities, viz. long-term fertilization and other treatments (Cassman et al. 2016). In brief, all major biomes on earth are interlinked and affect each other (Fig. 4.2). In a recent study of 189 global top-soil samples around the world, Bahram et al. (2018) have reported for the first time an opposite biogeographic trend for bacteria and fungi, simultaneously taking into account interkingdom antagonistic

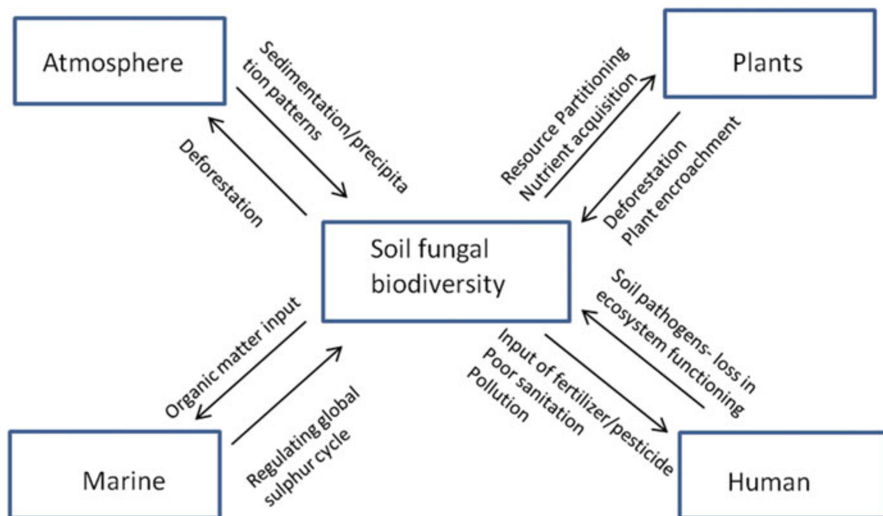

Fig. 4.2 Flow diagram illustrating the link between different biomes

interactions in structuring microbial community. The soil C-N ratio and precipitation were the important deterministic factors for fungal richness unlike bacteria where soil pH was the best predictor of diversity. Fungal communities were found to be mostly structured according to nutrient availability on account of selective substrate utilization and greater energy demands.

4.4.2 Mycobiome of Indoor Environment

Indoor microbes are gaining considerable attention as they result in detrimental effects on the inhabitants and their health. People spending time indoor are much more likely to develop sick building syndrome which is characterized by fatigue and discomfort (Haleem Khan and Mohan Karuppayil 2012), hay fever, allergic rhinitis (Husman 1996) and some neuropsychiatric disorders resulting in lack of concentration (Drappatz et al. 2007). Within the built environments, first attempts were made to study the distribution of microorganism with a major focus on bacteria on door handles from building at the University of Waterloo; a highly variable community was reported with prevalence of *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (Ross and Neufeld 2015). Exposure to fungal spores or mycelia present in the indoor building environment such as prefabricated gypsum board may act as an allergen and cause diseases, strongly inducing IgE sensitization against pathogenic forms (Nambu et al. 2009). Looking at its vital importance, a systematic attempt has been made by the Oslo Mycology Group (University of Oslo) in close collaboration with the Norwegian consulting firm Mycoteam AS with an aim to

provide a basic understanding of the indoor mycobiome of more than 200 buildings for the benefit of humankind (https://cordis.europa.eu/project/rcn/209177_en.html).

While investigation of microbiome associated with built-in environments has been characterized by several authors (Ross and Neufeld 2015; Wilkins et al. 2016), complementary studies have now laid emphasis on mycobiome (Tong et al. 2017) as also towards their cross-domain interactions. A concept of bacterial and fungal fingerprint has also emerged as indoor surfaces share major resemblance with the skin of household occupant; abundance of *Propionibacterium*, *Staphylococcus*, *Corynebacterium* and *Streptococcus* has been reported in the former (Wilkins et al. 2016), whereas in case of fungi, *Malassezia, Aspergillus* and *Cladosporium* are the prevalent genera (Tong et al. 2017). Household and air currents appear to be stronger factors then ventilation strategy, room type and human occupancy in defining the composition and structure of the indoor airborne communities. Therefore, use of an air purifier and frequent opening of windows can minimize fungal sensitization of allergic children (Nambu et al. 2009). There is a positive correlation between bacterial and fungal community diversity and composition under such environmental situations. Fungi appear to disperse farther compared to bacteria during winter; also, bacteria show dispersal limitation at a local scale (Tong et al. 2017).

Recent study of mycobiome also includes isolated systems like inflatable lunar/ Mars analogue habitat (ILMAH) which mimics International Space Station (ISS) conditions and could affect health of future immunocompromised astronauts; predominant cultivable fungal species found in ILMAH were species of *Epiccocum*, *Alternaria, Pleosporales, Davidiella* and *Cryptococcus,* which are significantly affected by human presence (Blachowicz et al. 2017).

4.4.3 Plant Mycobiome

Plant microbiome is considered to be an extension of host genome which determines the health and productivity of plants (Turner et al. 2013). It is quite complex and dynamic. The microbiome associated with phyllosphere, rhizosphere and endosphere has been found to be distinct for the same plant. Moreover, the taxonomic composition of microbiomes of same niche of different plants can be quite different. The abiotic conditions, viz. temperature, pH and moisture, have significant effect upon microbiome either directly or indirectly through the host. The other external factors can be affected by the cross-talk between the 'above- and below-ground plant tissues' (Turner et al. 2013). The plant immune system also plays a role in structuring the microbiome. Mycobiome may either exert negative impact on plant by being pathogenic or positive effect by increasing the stress tolerance of plants, decreasing herbivory or reducing infection of plant pathogens or providing nutrients (Arnold et al. 2003; Wilkinson et al. 2000; Newton et al. 2010).

The structure of rhizosphere microbiome is influenced primarily by the root exudates (Badri et al. 2013). The physical and chemical properties of soil have been found to be significantly correlated with the change in the microbiome

(Schappe et al. 2017). *Proteobacteria* (especially *Alphaproteobacteria* and *Betaproteobacteria*) are usually found to be the dominant community. *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia* and *Acidobacteria* are the major groups inhabiting the rhizosphere (Turner et al. 2013). Rhizosphere fungi provide nutrients as well as antagonize plant pathogens (Ehrmann and Ritz 2014). *Ascomycetes* and *Basidiomycetes* have been reported to be the dominant rhizosphere fungi (Bai et al. 2015). The significant increase in the abundance of *Thelebolus* and Mortierellales was observed in soybean rhizosphere after continuous cropping (Bai et al. 2015). Mycorrhiza forms a connecting link between plant and soil, constituting 'mycorrhizosphere' (Pagano et al. 2017). The mycorrhizal hyphae are influenced by bacteria, fungi, nematodes, protozoans and other organisms.

Phyllosphere has a large exposed surface area and is connected to air microbiome, thus forming the air-plant interface (Berg et al. 2014). It is considered to be nutrient poor as compared to rhizosphere. The leaf structure is known to influence the microbiome. It constitutes a more dynamic environment as compared to rhizosphere, where resident microflora is subjected to wide fluxes in temperature, radiation and moisture. The precipitation and wind contribute to the temporal variation in the microbiome (Lindow and Brandl 2003). *Proteobacteria* (*Alphaproteobacteria* and *Gammaproteobacteria*) is found to be the dominant phylum. *Actinobacteria* and *Bacteroidetes* are the other commonly found phyla (Bodenhausen et al. 2013). The phyllosphere fungi are considered to be the important drivers of plant communities (Bradley et al. 2008). They influence the dynamics of phyllosphere bacteria (Suda et al. 2009). The culture-based approaches excluded biotrophic species, but with the advent of culture-independent approaches, a complete description of phyllosphere fungal community is obtained (Jumpponen and Jones 2010). *Ascomycetes* have been reported to be the dominant fungi of phyllosphere. Cordier et al. (2012a, b), reported ascomycetous yeast *Taphrina carpini,* ascomycetous black yeast *Venturia hanliniana* and ascomycetous saprobe *M. flageoletiana* as the three most abundant fungi in European beech.

Endosphere is considered to be harbouring the subpopulation of rhizosphere microbiome though both are found to be significantly different (Compant et al. 2010). They are generally considered to be non-pathogenic but may include latent pathogens (Monteiro et al. 2012). They usually enter at lateral root junctions via cracks; however, they also produce small amounts of cell wall-degrading enzymes (Compant et al. 2010). The number of endophytes is generally found to be lower in aerial parts than in roots. The metagenomic analysis revealed Proteobacteria (α, β and γ) and Firmicutes as the common endophytes (Sessitsch et al. 2012). *Azoarcus*, *Burkholderia*, *Gluconacetobacter*, *Herbaspirillum* and *Klebsiella* sp. are the commonly isolated endophytes from non-legumes, especially grasses (Compant et al. 2010). Gang et al. (2017) isolated a total of 79 endosphere and 440 rhizosphere fungi from the first-class endangered wild plant *Cypripedium japonicum* which were identified to be 65 genera and 119 species based on molecular characteristics. *Trichoderma* was found to be the most dominant in both endosphere as well as

rhizosphere. *Mortierella, Hypocrea* and *Penicillium spp.* were found to be the other dominant fungi.

4.5 Conclusions

Fungi constitute a major portion of various ecosystems, but their greatest diversity is present in terrestrial habitats. Like plant and animal kingdoms, fungi also exhibit biogeographical patterns depending upon latitudes, climate, evolutionary relationships and dispersal limitation affecting at local and global scales. Comparative studies of bacterial and fungal domain prepare a strong fundamental platform to study interkingdom interactions and a clearer picture of the structure of microbial communities and their functions. There are known direct and/or indirect benefits conferred by fungi towards the major biomes on earth which result in a better and clearer understanding of the modulation of immunity in humans, productivity of plants, climate change scenario or presenting an altered biogeography of soil microbial community.

Next-generation sequencing has uncovered fungal diversity at greater depth which was previously unknown. Powerful and more robust technologies have generated an expanded data which have built up a strong foundation so that the importance of mycobiome can no longer be underestimated. This has, however, led to renewed interest in understanding the functional and interkingdom interactions rather than mere cataloguing of the microbial diversity, their therapeutic role and use as diagnostic markers in human disease. Recent efforts also clearly indicate greater role of mycobiota in diseases with complex aetiology such as IBD and genetic and psychiatric disorders which were previously not linked with fungi. Increasing knowledge on gut mycobiome and its role in maintaining gut flora homeostasis might provide insights into therapeutic intervention leading to better understanding of several neurobiological diseases. Combined knowledge of host genetics and mycobiome along with its interaction with other microorganisms might be helpful in developing personalized medicine. Similarly, studies from indoor-built environment provide important insights into controlling building design and fungal allergens besides providing a sound base to study the complex relationships between indoor fungal exposure and human health. Studies dealing with terrestrial ecosystem are now directed towards holobiont characterization of fungi from plants which show beneficial effect and variation of individual microbial taxa across tissue-level niche and its comparative demarcation with the bacterial counterparts.

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Part II

Microbes in Normal and Extreme Environments

Marine Microbial Diversity for Sustainable Development

5

Anil Kumar Pinnaka and Naga Radha Srinivas Tanuku

Abstract

The marine ecosystem is the largest habitat on our planet Earth. Microbes encompass the huge diversity and play a vital and frequently inimitable task in the operation of the ecosystem and in preserving a sustainable ecosystem. Marine microbes belong to three domains of life such as "Archaea," "Bacteria," and "Eukarya." Both prokaryotic domains are highly abundant in the oceanic ecosystem. Apart from these, viruses were tenfold more abundant than all microbes in the marine environment. Microorganisms participate in sustaining the vibrant balance and uprightness of the environment, which is vital because the sustained continuation of life is reliant upon the continued, microbially mediated alteration of substances in the marine habitats and most of the biogeochemical processes in the marine ecosystem engross microbes, either directly or indirectly. Till now, the scientific community still does not completely understand microbial diversity and its significance on environmental processes. It is a wrong perception that microbes only cause diseases, because there are several constructive roles they play in marine habitats, and, therefore, it is necessary to intensely investigate the marine microbes as they aid immensely in sustainable development in human welfare. The chapter discusses the distribution and diversity of diverse microorganisms, their unique roles in the oceans in ecosystem services, environmental sustainability, human health, and achieving sustainable growth.

Keywords

Microbial diversity · Biogeochemical cycles · Marine ecosystem · Human health · Sustainable development · Global environmental change · Societal benefits

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5.1 Introduction

Microorganisms are very small and cannot be viewed through our naked eyes as they are smaller than 0.1 mm in size and as small as 0.02 μm. Marine ecosystem is the largest biome on the globe, spanning around 70% of our planet, comprises huge biomass, and has a considerable part in the biogeochemical cycles. Life on our planet perhaps evolved from microorganisms in the marine habitat, and majority of the marine biomass is dominated by microorganisms such as Archaea, Bacteria, fungi, protists, and viruses. They are able to thrive in all marine habitats, from the top micro layer of the ocean surface to several kilometers below the ocean sediments, and they are viable and multiply in diverse inhospitable environments such as supercooled brine channels to near-boiling waters and anoxic conditions to withstanding high pressure. Apart from their profusion in the ocean sediments and waters, they are diverse and part of a vast array of anabolic and catabolic processes and involved in almost all biogeochemical processes happening in the oceans around us. They are tiny, single-celled (mostly) and account for around 98% of the total oceanic biomass. Marine microbes were neglected so far, but recent molecular developments in marine molecular ecology, omics (metagenomics, metatranscriptomics, metaproteomics, and metabolomics), and ecological modeling showed that these represent most significant group on our globe in terms of genetic and structural diversity, and recent multidisciplinary studies have discovered microbes with unique functions in several biogeochemical cycles of carbon, nitrogen, silica, iron, phosphorus, sulfur, and several trace elements. So far, ~2 million species have been discovered, but many ecologists believe that there are ~13 million species on our Earth and further estimate between 3 and 100 million. They are part of primary production either by using sunlight or reduced chemical as an energy source. Oceanic photic zone is the place where half of the primary production occurs and the CO_2 generated by biological and anthropogenic activities is consumed. Oceanic phytoplankton produce >50% of oxygen on our planet. Several heterotrophic microbes consume the organic matter generated during primary production and mineralize the same, which is further available to the primary producers again. They are part of cloud generation, fishery production, and host to several bioactive molecules. So marine microbes are the base of life on our Earth and are of critical importance for habitable and sustainable function. The vast microbial diversity provides principally untouched quantity of hereditary information, bioactive substances, and biological matters which can bring significant profit and utility of public attention (progress in hydroponics applications, halieutics, therapeutic management, and the provision of energy and for the expansion of trade goods and practices). They are part of several processes which maintain the marine ecosystem in a sustainable way. Hitherto, regardless of the clear significance of oceanic microorganisms and the foremost prospects they provide, only small part is recognized concerning oceanic microbial heterogeneity, the vast collection of microbial diversity, and their environmental roles and relations, and huge part (90–99%) of oceanic microbes are uncultivated under typical lab environments and their multiplication and physiology cannot. They play a crucial function to guarantee a

sustainable provision of marine food substances by engaging the important base of trophic rank in food webs and contributing answers for adapting hydroponics practices, treating pollutants, and waste treatment but are influenced due to global change and might support or alleviate weather modification. Oceanic microbes can provide a huge quantity of goods and services that support human well-being, such as the provision of food, fuel, and building materials; stabilization and restraint of the Earth's climate; cleansing of air and water; moderation of floods, droughts, temperature extremes, and wind forces; maintenance of genetic resources as inputs to medicines and other products; and cultural, frivolous, and aesthetic benefits. They have a myriad prospective for providing services and goods for the benefit of humanity which is not oppressed to some noteworthy level. The world of marine microbes to be find out, recognize their potential and put them to good use.

5.2 Marine Microbial Diversity and Distribution

5.2.1 Marine Bacteria

Chemoorganotrophic bacteria are the majority of copious microorganisms in the marine ecosystem, and the entire biosphere after viruses and their biomass is also significant. Dissolved organic matter (DOM) is utilized by many microbes such as cyanobacteria, eukaryotic photosynthetic planktonic microbes (Mulholland et al. 2002), protozoans, and possibly still bigger eukaryotic microbes, but significant proportion of DOM utilization is by heterotrophic bacteria. The dominance in DOM utilization is due to their size (0.1–2 μm), their surface area to volume ratio, and also their metabolism compared to other microbes cited above. The abundance of microbes increases with their decreasing size in the marine ecosystems (Table 5.1). This is also true with respect to their metabolism. Heterotrophic microbes will be distributed throughout the water column and also in sediments. Due to large biomass, they have some limitation in their growth rates. Their multiplication in natural habitat is generally more than a day and even longer such as weeks or months in oligotrophic conditions when calculated for the whole population, but in the laboratory, it is half an hour or more. Chemoorganotrophic bacteria from marine ecosystem which are part of "microbial loop" coined by Azam et al. (1983) did not receive much attention in the scientific community till early 1980. Earlier reports underestimated that bacterial abundance and species richness in diverse marine environments mainly depend on traditional cultivation and microscopic staining techniques (Jannasch and Jones 1959) but after the development of DNA-based staining techniques and use of fluorescence microscopy revealed the broad discrepancy in cultivable bacteria vs total microbial counts in the given sample (great "plate count anomaly") (Hobbie et al. 1977; Porter and Feig 1980; Zimmermann and Meyer-Reil 1974) and showed that earlier studies less quantified microbial abundance as low as three times. Whitman et al. (1998) showed an average of 10^9 cells per liter (fluorescence-based) of seawater compared to 10^5 (culture based). Jørgensen and Boetius (2007) showed cell abundances of 10^8–10^9 per

Table 5.1 Microbial diversity and distribution in the marine ecosystem

S. no.	Microbes	Size range (in μm)	Diversity	Abundance (log of cells/l)	Distribution	Metabolism
1.	Bacteria	0.1–2[a]	*Proteobacteria*, *Bacteroides*, *Firmicutes*, *Actinomyces*, *Chlorobi*, *Spirochetes*, *Flavobacteria*, *Planctomyces*, *Chlamydiae*, *Chloroflexi*, *Thermotogae*, *Aquificae*, *Acidobacteria*, *Fibrobacteres*, *Deferribacteres*, *Lentisphaerae*, *Verrucomicrobia*, *Deinococcus-Thermus*, *Elusimicrobia*, *Fusobacteria*, *Gemmatimonadetes*, *Nitrospirae*, *Synergistetes*, *Tenericutes*, and *Thermodesulfobacteria*	10^8–10^9	Throughout the water column and sediment (more abundant in photic depth)	Photoheterotrophic, chemoautotrophic, chemoheterotrophic, mixotrophic
2.	Cyanobacteria	0.5–10	*Prochlorococcus*, *Synechococcus*, *Synechocystis*, *Merismopedia*, *Dermocarpella*, *Pleurocapsa*, *Myxosarcina*, *Chroococcidiopsis*, *Oscillatoria*, *Spirulina*, *Lyngbya*, *Microcoleus*, *Nostoc*, *Calothrix*, and *Trichodesmium*	10^6–10^8	Photic depth in water column and in coastal sediments	Photoautotrophic, photoheterotrophic
3.	Archaea	0.1–15	Crenarchaeota, Euryarchaeota, Thaumarchaeota, Korarchaeota, Nanoarchaeota, Pacearchaeota, and Parvarchaeota	10^8–10^9	Throughout the water column and sediment (more abundant in deeper depths)	Chemoautotrophic, chemoheterotrophic

4.	Autotrophic micro eukaryotes	<3–100	Bacillariophyta, Chlorophyta, Haptophyceae, Alveolata, Stramenopiles, choanoflagellates, and Acantharea		Photic depth in water column and in coastal sediments	Photoautotrophic, photoheterotrophic
5.	Heterotrophic micro eukaryotes	1–100	Protozoa, Actinopoda, Retaria, Cercozoa, Ciliophora, Dinophyceae, larval stages of Cnidaria, Ctenophora, Rotifera, Platyhelminthes, Nematomorpha, Nemertea, Annelida, Mollusca, Crustacea, Chaetognatha, and Chordata	10^4–10^5	Throughout the water column and sediment (more abundant in photic depth)	Chemoheterotrophic
6.	Fungi	3–>100	*Ascomycota*, *Basidiomycota*, *Blastocladiomycota*, *Chytridiomycota*, *Deuteromycota*, *Glomeromycota*, *Microsporidia*, and *Zygomycota*		Abundant in mangrove water and sediment, parasites on other eukaryotes, and as symbionts (lichens)	Saprobic, parasitic, decomposers
7.	Viruses	0.02–0.2	*Baculoviridae*, *Rhabdoviridae* , *Nimaviridae*, *Phycodnaviridae*, *Corticoviridae*, *Tectiviridae*, *Myoviridae*, *Podoviridae*, *Siphoviridae*, *Paramyxoviridae*, *Microviridae*, *Parvoviridae*, *Birnaviridae*, *Leviviridae*, *Orthomyxoviridae*, *Nodaviridae*, *Reoviridae*, *Picornaviridae*, *Lipothrixviridae*, *Cystoviridae*, *Dicistroviridae*, *Bunyaviridae*, *Marnaviridae*, *Caliciviridae*, *Herpesviridae*, *Coronaviridae*, *Iridoviridae*, *Papovaviridae*	10^9–10^{10}	Throughout the water column	Lytic, lysogenic

[a]*Thiomargarita namibiensis* is up to half a millimeter long, and *Epulopiscium fishelsoni* reaches 0.7 mm which is observable even with the naked eye

gram of marine surface sediments and around 10^5 in the greatest depths of the subsurface seabed. Although the common feature of DOM utilization, marine heterotrophic bacteria are very diverse, and their distribution depends on the environmental conditions prevailing at that time. There were several marine bacteria cultured so far, but their wide distribution is evident from molecular methods and belongs to a number of phyla *Proteobacteria*, *Bacteroides*, *Firmicutes*, *Actinomyces*, *Chlorobi*, *Spirochetes*, *Flavobacteria*, *Planctomyces*, *Chlamydiae*, *Chloroflexi*, *Thermotogae*, *Aquificae*, *Acidobacteria*, *Fibrobacteres*, *Deferribacteres*, *Lentisphaerae*, *Verrucomicrobia*, *Deinococcus-Thermus*, *Elusimicrobia*, *Fusobacteria*, *Gemmatimonadetes*, *Nitrospirae*, *Synergistetes*, *Tenericutes*, and *Thermodesulfobacteria*. Heterotrophic bacteria were more abundant in the photic depth where more DOM is available (from terrestrial sources especially in coastal regions, in situ production from phytoplankton, partial grazing by zooplankton, and viral lysis). Bacteria are very diverse in their metabolism, i.e., they can grow photoheterotrophically, chemoautotrophically, chemoheterotrophically, and also mixotrophically. In molecular phylogenetic studies using ribosomal RNA genes, a major group of marine microbes were discovered, and they belong to a very discrete evolutionary lineage in the *Alphaproteobacteria* which is named as SAR11 cluster (Giovannoni et al. 1990) and is very dominant, comprising around 33% of several euphotic zone environments, and covers around 25% in mesopelagic bacterial assemblages (Morris et al. 2002). The proteorhodopsin is a pigment, considered as vital in providing energy to bacterial cells through light-driven proton pump. Zinger et al. (2011) studied a large molecular dataset (96,00,000 V6-rRNA gene sequences of bacteria) from a large number of samples (509) that cover the worldwide ocean's top to bottom layer and revealed a great difference in bacterial assemblages from pelagic to benthic, at every taxonomic stages, and distribute, 10% bacterial groups (using 3% cutoff). Based on primary productivity, continental discharges, anthropogenic pressure, and ecological limitations, for instance, oxygen accessibility impacts the diverse microbial communities in total water column, shoreside and high seas, and anoxic and oxic marine environments (Zinger et al. 2011). Marine habitats are assorted and dynamic in nature as apparent from lofty unevenness of bacterial community composition explicit to coastal and hydrothermal vent environments, and at the same time, marine biological export flux showed the correlation between pelagic and benthic bacterial diversity with surface water production and bacterial distribution in marine habitats depends on varied physical mixing which results in higher dissimilarity in benthic diversity with increasing depth compared with pelagic diversity (Zinger et al. 2011). There were several uncultured bacteria with distinct clades identified recently using molecular methods which include SAR86, SAR202, SAR406, Clade-1a, surface1, clade-1b, NS9, MGII, DHVEG-6, WCHB1-41 (Lloyd et al. 2018), SAR116, Marine Group A, and Marine Group B. Yilmaz et al. (2016) disclose 92 marine clades that have been so far unrecognized.

5.2.2 Marine Cyanobacteria

Cyanobacteria, also called as blue-green algae, have been found to harbor pigments. They are photosynthetic bacteria harboring chlorophyll *a* which gives greenish color and carotenoid phycocyanin which provides blue color to the organism. Cyanobacteria were widely distributed in several aquatic habitats where light source is available, such as several terrestrial habitats (rivers, ponds, fields, lakes, canals, wastewater bodies, polar or cold habitats) and marine habitats (estuaries, mangroves, coastal waters, coastal surface sediments, open ocean waters). Marine cyanobacteria were highly diverse and belong to different genera *Prochlorococcus*, *Synechococcus*, *Synechocystis*, *Merismopedia*, *Dermocarpella*, *Pleurocapsa*, *Myxosarcina*, *Chroococcidiopsis*, *Oscillatoria*, *Spirulina*, *Lyngbya*, *Microcoleus*, *Nostoc*, *Calothrix*, and *Trichodesmium*. Cyanobacteria were abundant in diverse marine habitats and range from 10^6 to 10^8 cells per liter. Marine microbiologists studying the diversity from more than a hundred years, however, noticed huge numbers of marine *Synechococcus* (Waterbury et al. 1979) and *Prochlorococcus* (Chisholm et al. 1988) in the recent past. Marine cyanobacteria harbor convenient markers (pigments) which help microbiologists to quantify them easily compared to other marine bacteria. Because of the unique pigments, it becomes possible to study *Synechococcus* using fluorescence microscope and flow cytometer and *Prochlorococcus* with flow cytometer. Both organisms were cultured in the laboratory, and several of their physiological and biochemical properties were studied, and genome sequences were also available (Dufresne et al. 2003; Rocap et al. 2003; Palenik et al. 2007). *Synechococcus* are cocci in shape and 0.9 μm in diameter; possess chlorophyll *a*, phycocyanin, and phycoerythrin; and are cosmopolitan in distribution. Some species can fix dinitrogen. *Synechococcus* were abundant in coastal water. Cells of *Prochlorococcus* were also coccus in shape and of 0.7 μm in diameter, possess chlorophyll *a* and *b* and phycobilins, and are distributed in oceanic gyres and were less abundant in coastal waters compared to *Synechococcus* and does not fix dinitrogen. Both of them were not noticed in cold, high-latitude oceans. Marine cyanobacteria were highly abundant (~90%) in terms of total phytoplankton, especially in the nutrient-poor oligotrophic marine ecosystems. Both of these were contributing largely to the phytoplankton numbers and their primary production in the marine ecosystem but were smaller than eukaryotic phytoplankton. Based on phylogenetic analysis, cyanobacteria belong to the domain Bacteria (Hoiczyk and Hansel 2000), and like other prokaryotes, they are without nucleus and resemble Gram-negative bacterial cell wall and membranes, but as per their function, they are part of marine phytoplankton community as they are primarily photosynthetic and employ sun radiance power to fix carbon dioxide (CO_2) by analogous mechanisms (Calvin cycle) like in eukaryotic members. Recent studies showed that *Prochlorococcus* and *Synechococcus* were photoheterotrophic and can

incorporate or assimilate leucine/methionine (Zubkov and Tarran 2005; Michelou et al. 2007). Marine cyanobacteria are distributed in photic depths. From the recent metagenomic studies (V6 rRNA region sequence), it is evident that the major tag sequence belongs to phytoplankton community, a member of the *Prochlorales* (*Cyanobacteria*), and was very close to the cultivar *Prochlorococcus marinus*. *Synechococcus* and *Prochlorococcus* together dominate the oceanic ecosystems with cell numbers ranging from 10^5 to 10^6 per ml in the northern oligotrophic gyre (Heywood et al. 2006; Scanlan et al. 2009) and amounting half of marine primary production (Li 1994). These represent a primordial entity of microbes, which is evident from the fossil records of stromatolites which might date back from 2.1 Ga (Lane 2010). Cyanobacteria oxygenate the Earth's atmosphere from past 25,00,000 years which results in the origin of plants and animals around 5,00,000 years ago.

5.2.3 Marine Archaea

Archaebacteria an old term shows the common features of Archaea and Bacteria. Archaea comprise a domain of unicellular microorganisms and are prokaryotes as they are without nucleus and membrane-bound organelles. Archaea and bacteria were not distinguishable based on their simple microscopic features like similar shape and size, but a few encompass unique shapes like plane and square appearance like *Haloquadratum walsbyi* (Stoeckenius 1981). Archaea are comprised exclusively of microorganisms. Even though Archaea and Bacteria do not differ much in morphological features, they demonstrate a huge array of metabolic capacities. Although they are morphologically similar, they possess unique features that make them different from bacteria such as lack of peptidoglycan layer in cell wall and ether-linked lipids in contrary to ester-linked lipids in bacteria and similar enzymes involved in translation and transcription, genes and several metabolic pathways of eukaryotes and differ with them in terms of using more energy sources vary from organic substances (sugars), inorganic compounds (ammonia, hydrogen gas, and metal ions). Haloarchaea employ solar radiation for energy generation, some can fix carbon dioxide, and some members fix dinitrogen (Leigh 2000). Archaea metabolize photoheterotrophically by using sunlight and organic carbon (*Halobacterium*), chemolithoautotrophically or heterotrophically by using inorganic/organic compounds (*Ferroglobus*, *Methanobacteria*, or *Pyrolobus*), and chemoheterotrophically by using organic compounds (*Pyrococcus*, *Sulfolobus*, or *Methanosarcinales*). Archaea are less narrowly associated evolutionarily to bacteria than to eukaryotes. Archaea were initially isolated from harsh environmental conditions, for instance, brines and hot springs, and they were thought to be extremophiles. Based on the pyrosequencing analysis, *Crenarchaeota* members are more abundant, and marine microbial ecologists were in the view that all these members represented extremophiles and were later found that to be distributed in diverse habitats such as soils, human microbiota, ocean habitats, marshlands, etc. (DeLong 1992; Simon et al. 2000; Fuhrman et al. 1992). *Halobacterium* can grow in salt concentrations more than 25% (Valentine 2007), and *Methanopyrus kandleri*, a

thermophile, can reproduce at 122 °C (Takai et al. 2008). Earlier studies overestimated the bacterial abundance in the marine ecosystems using fluorescent dyes as both prokaryotes of comparable shape and size. Identification of oceanic archaea was possible after the advent of molecular techniques, but their huge number and their function in the marine ecosystems were not known to the scientific community until recently (Karner et al. 2001; Ingalls et al. 2006), and these studies found that they are cosmopolitan in distribution and their abundance is nearly equal or slightly higher in deeper depths of the ocean waters. Some of the oceanic archaea are chemolithoautotrophic in their metabolism and oxidize ammonium as an energy source (chemolithotrophy) to fix CO_2 (chemoautotrophy). Till recently there were no archaea identified as pathogen or parasite, but they are often mutualists or commensals. Herndl et al. (2005) showed that abundances of crenarchaeotal members are more than bacteria under 100 m deep in the marine waters and they take part in active metabolism and commit to the marine organic matter utilization. Galand et al. (2009) showed that Euryarchaeota members dominate in the underneath water masses of Arctic (cavernous Atlantic Layer in the central Arctic Ocean), and these were second most abundant in archaeal pyrotags. And some archaeal pyrotags such as methanogens, Methanosarcinales and *Archaeoglobus* and *Methanococcus* were abundant in Lost City Hydrothermal Vents and sulfide chimneys, respectively. And in the Lost City Hydrothermal Vents, Methanosarcinales is the single archaeal group dominant there, and within the biofilm large physiological diversity was evident from morphological features, specific gene diversity, genes concerned in diazotrophy, methanogenesis, and anoxic oxidation.

5.2.4 Marine Eukaryotic Phytoplankton

5.2.4.1 Marine Micro- and Nano-eukaryotic Phytoplankton

Fixation of carbon using Calvin cycle or the conversion of inorganic carbon (CO_2) to a “preset,” organic carbon is an initial part of the carbon cycle. In oceanic waters, fixation of carbon is exclusively done by free-floating microbial plants, also called as phytoplankton, except in few coastal habitats like mangroves and tidal marshes in which plant production governs and in superficial aquatic habitats benthic algae dominate primary production (Behringer and Butler 2006; Segal et al. 2006; Gattuso et al. 2006). In aquatic and terrestrial ecosystems, phytoplankton and plants are part of significant primary production, which share the same mechanism, the Calvin-Benson-Bassham cycle. In contrary to the land plants, marine phytoplankton harbors specific pigments called the carotenoids which are “the light-harvesting pigments.” These pigments delineate some of the taxa such as alloxanthin and monadoxanthin that are unique to Cryptophyta; dinoxanthin, peridnin, peridininol, and pyrrhoxanthin are unique to Dinophyta and lutein in Chlorophyceae. In some cases some pigments were dominant in some groups such as fucoxanthin in diatoms. As a result of high concentration of carotenoids, most of the marine phytoplanktons are not green.

In many marine ecosystems, eukaryotic phytoplankton community are also a significant portion of total phytoplankton biomass especially in coastal waters, estuarine, and mangrove habitats where large species (10–100 μm) dominate, and their identification is relatively easy due to their unique sizes and shapes, apart from their respective pigments which utilize sunlight such as diatoms, dinoflagellates, coccolithophorids, silicoflagellates, and raphidophytes. Some of them are known for their significance part in coastal waters, when they form dense blooms during the spring season. Based on pyrosequencing analysis of rRNA gene sequences, dinoflagellates were abundant among the majority of eukaryotic microbial communities, and this shows that both phototrophic and heterotrophic representatives might be accountable for detrimental algal blooms, creating them economically and environmentally vital. The distribution of these large phytoplankton communities is mostly in coastal habitats and also in photic depths only but contributes much to the biological export flux.

Gast et al. (2006) first identified an unclassified dinoflagellate within the *Karenia/Karlodinium* group (highly abundant tag) in the eukaryotic datasets showing 100% similarity with the gene observed in the Ross Sea, Antarctica. The cell abundance reaches 29,000 l^{-1} in some cases, and this pyrotag is furthermore observed in the Arctic, Atlantic, and Pacific Oceans, the Black Sea, and the Framvaren Fjord in Norway. However it is not known that this pytotag belongs to the similar cosmopolitan species or intimately linked ecotypes that extend over the planet Earth.

5.2.5 Marine Picoeukaryotic Phytoplankton

Because of the advent of environmental polymerase chain reaction, cloning, sequencing, and environmental metagenomics approaches reveal a vast unexplored diversity of phytoplankton community present in oceanic waters which are of picoplankton size and were part of the eukaryotic picoplankton and were found in all major algal groups (Chlorophyceae, Pedinophyceae, Prasinophyceae, Trebouxiophyceae, Cryptophyceae, Prymnesiaceae, Bacillariophyceae, Bolidophyceae, Chrysophyceae, Dictyochophyceae, Eustigmatophyceae, Pelagophyceae, and Pinguiophyceae). In contrast to large-sized phytoplankton, the pico-fraction is tough to recognize by conventional microscopic observations, because of their small size and not having unique characteristics. This shows the presence of an astonishing biodiversity of oceanic picoeukaryotes even though their impending role in major biogeochemical processes is mostly unidentified. In the pyrosequencing analysis, several tags were noticed among the top 20, the majority of copious eukaryotic microbes were constituent of the picoeukaryotic community that statistically dominate, but their 18S rRNA genes with minor copy numbers (Díez et al. 2001; Moon-van de Staay et al. 2001; López-García et al. 2001). The first picoeukaryote *Ostreococcus tauri* identified from the Thau lagoon in France is the smallest photosynthetic eukaryote known till now (Courties et al. 1994). Recent environmental genomic analysis noticed the worldwide presence of the omnipresent oceanic picoeukaryotes such as *Micromonas pusilla* and *Ostreococcus* spp. which

denotes the presence of ecotypes. One of the smallest known for a free-living eukaryote *Ostreococcus tauri* comprises *c*. 12.6 Mb genome (8166 predicted genes) with the distinctive enzymes part of C_4-like photosynthesis.

5.2.6 Marine Eukaryotic Zooplankton

Plankton are defined as organisms drifting in aquatic habitats such as lakes, ponds, canals, rivers, seas, and oceans. Heterotrophic plankton are named as zooplankton and are typically microscopic, but a few are bigger and noticeable to the naked eye such as jellyfish. Zooplankton were classified based on size range from pico to macro and mostly pico-, nano-, and micro fraction comprises protistans and macrofraction comprises large metazoans. They belong to two different life cycles holoplanktonic (plankton during complete life cycle) and meroplanktonic (live as plankton in part of their life cycle and after maturation they live as nekton/sessile, benthic subsistence) and comprises locomotory organs such as flagella and cilia, although they belong to plankton lifestyle; this is to avoid predation and to get prey. Microbial zooplankton generally comprises protists (Protozoa, Actinopoda, Retaria, Cercozoa, Ciliophora, Dinophyceae) and the larval stages of several higher taxa (Cnidaria, Ctenophora, Rotifera, Platyhelminthes, Nematomorpha, Nemertea, Annelida, Mollusca, Crustacea, Chaetognatha and Chordata). Their abundance varies from 10^3 to 10^5 cells/l of marine water and was disseminated all through the pelagic region but more abundant in photic depth. They are primarily chemoheterotrophs. Protists from marine ecosystem are mostly single celled and highly different, in terms of morphology and function. Some of the marine protists are part of phytoplankton assemblage and contribute to primary production, and most others are chemoorganotrophic and prey on microbes by phagotrophy (swallow up the prey into food vacuole for digestion). And some of them were mixotrophic carrying both photosynthesis and grazing (Adolf et al. 2006). They feed on the prey in three different types, i.e., filter, diffusive, and raptorial feeding, and do not have mouth but ingest prey mostly by phagocytosis and digest in food vacuole inside the cell. Some examples of mixotrophic flagellates are *Micromonas pusilla*, *Prymnesium*, and *Myrionecta rubra*.

5.2.6.1 Microzooplanktonic Protists (20–200 μm)

Tintinids, a type of ciliate with intricate shells (loricate), catch the attention of earlier marine ecologists (Sherr and Sherr 2000). The advent of more advanced methods resulted in the identification of several “naked” or “aloricate” ciliates. These microzooplankton protists are abundant in the marine waters and are pretty imperative as herbivory (grazers of phytoplankton) and also predate flagellates which prey on cyanobacteria, picoeukaryotes, and even bacteria. Due to this predation, ciliates become link between picoplankton and zooplankton which completes the “microbial loop.” McManus and Fuhrman 1986 found that *Laboea spiralis* (mixotrophic ciliate) fix CO_2 using chloroplasts from ingested phytoplankton (partially digested). The name “ciliate” is given to these groups based on their morphology and motility, but their environmental function in the marine ecosystem varies widely. Ciliates belong

to micro fraction exclusively, and some examples of ciliates include *Dictyocysta mitra*, *Fabrea salina*, *Laboea strobila*, *Strombidium inclinatum*, and *Trichodina* spp. Protists also comprise amoeboid and belong to nano, micro, and macro fractions, which are Acantharea, Phaeodaria (possessing shells of silica or strontium sulfate), and Foraminifera (possessing calcium carbonate shells).

5.2.6.2 Dinoflagellates (20–200 μm)

The dinoflagellates are a hefty collection of eukaryotic flagellate within the phylum Dinoflagellata. Most are marine plankton, and their populations are disseminated depending on sea surface temperature, salinity, or depth. These attract the microbial ecologists based on their diverse size and shapes and the size ranges from nano fraction (unarmored members, like *Gymnodinium* with 8–15 μm) to meso fraction (*Noctiluca* which are 200–2000 μm). Dinoflagellates are generally armored with plates made up of cellulose (theca) with two groves to hold the two flagella (motility). Several members are photoautotrophs and fix CO_2 (*Gymnodinium*), others are heterotrophic and graze other microbes (*Noctiluca*), and some are mixotrophic (*Lingulodinium polyedrum* and *Akashiwo sanguinea*). Around half members are without pigments and probably heterotrophic. Heterotrophic dinoflagellates are of significant part of microzooplankton community and graze huge part of phytoplankton in the marine ecosystems (Strom et al. 2007). Most of them are microplankton, and some are very small and part of nanoplankton fraction, and many of them are bioluminescent mainly emitting blue-green light. Heterotrophic or mixotrophic dinoflagellates feed on their prey by different feeding modes such as phagocytosis, pedicle (a feeding tube used for to suck cytoplasm of prey), and pallium (feeding veil which connects to prey). Some of them are pathogens to higher marine biota and even humans. Dinoflagellates form bloom in marine waters where they discolor water called as red tide, which subsequently causes shellfish poisoning if people consume the same. The red color of the water is due to the high abundance of *Gymnodinium splendens* and *Gonyaulax polyhedra* which release strong neurotoxins also known as “soxotoxins.” Some are potential fish pathogens such as *Pfiesteria piscicida*, and some parasitize other dinoflagellates such as *Amoebophrya* (Sherr and Sherr 2000; Coats and Park 2002).

5.2.6.3 Nanoflagellates (2–20 μm)

Marine ecosystem harbors a huge number of small eukaryotes without chlorophyll and possesses one or more flagella named as “heterotrophic nanoplankton” (HNAN), or merely flagellates. Nanoflagellates are diverse taxonomically and perhaps in the same way varied in environmental and biogeochemical responsibility as that of other taxa. These were grazed on cyanobacteria, picoeukaryotes, and chemotrophic bacteria as the quarry have around the same dimension. Same-size prey is probably grazed by the similar predator, even though it may prefer between victim on the basis of chemical properties on the prey surface or “taste” (Strom 2000). Few heterotrophic nanoflagellates were named such as *Cafeteria roenbergensis* (*Bicosoecids*), *Massisteria marina*, *Bodo saltans* (*Bodonid*), *Paraphysomonas imperforata* (*Chrysomonad*), and *Monosiga* (choanoflagellate).

5.2.6.4 Picoeukaryotes (0.2–2 μm)

Using phylogenetic analysis (18S rRNA gene), it is understood that organotrophic marine picoeukaryotes were part of different lineages such as the Acantharea, Alveolata, choanoflagellates, and stramenopiles (Moon-van der Staay et al. 2001), and there are no cultured representatives in these groups till now. Massana et al. (2002) confirmed that new stramenopiles are bacterivorous using grazing experiments and were distributed in the pelagic waters, and recent fluorescent in situ hybridization studies noticed that picoeukaryotes are reasonably copious in the deep sea and few organotrophic descent are observed, unstratified, top to bottom (3000 m) (Moreira and Lopez-Garcia 2002). These heterotrophic picoeukaryotes belong to different genera such as *Caecitellus*, *Massisteria*, *Paraphysomonas*, *Picophagus*, *Phagomyxa*, *Pseudobodo*, and *Symbiomonas*. They are abundant in marine waters between 10^5 and 10^7 cells/l (Hall and Vincent 1990).

5.2.7 Marine Fungi

Marine fungi are a large group of eukaryotic microbes. Although marine ecosystems comprise yeasts and filamentous fungi, they are not ecologically important apart from the decomposition of arid debris from vascular plants in marshy habitats as their abundance is very low (Newell 2003); however, they contend with archaea and bacteria by utilizing DOM or particulate organic matter (POM) or detritus in the diverse marine habitats, but the bacteria dominate due to higher surface area to volume ratio. But filamentous fungi can utilize more POM in the dead plant material from salt marshes, with the help of hyphae which are capable to pierce and spread in arid, lifeless plant (which archaea and bacteria unable to do). Fungi are most important in the carbon cycle of terrestrial ecosystems due to the above reason. Obligate marine fungi grow solely in the oceanic ecosystems and at the same time entirely or infrequently sunken down in marine water. Diverse oceanic ecosystems support a huge diversity of fungi and are found in the niches such as estuaries, mangrove swamps, coastal waters, and oceanic deeper depths (in sediments). They are saprobic or parasitic on marine animals, algae, and dead wood. Marine fungi are classified into 1112 species which belong to 472 genera and belong to *Ascomycota*, Basidiomycota, *Chytridiomycota*, *Zygomycota*, *Blastocladiomycota*, asexual morphs of filamentous fungi, and marine yeasts *Ascomycota* and *Basidiomycota*. The major family of marine fungi (*Halosphaeriaceae*) comprise of 59 genera (141 species), while the majority of fallacious filamentous fungal genera belongs to *Penicillium*, *Aspergillus*, and *Candida* (yeast). Based on the small subunit rRNA gene sequences, 36 novel marine fungal descents were discovered, the preponderance among were chytrids and a few filamentous fungi, and the greater part of the species noticed belongs to basidiomycetous and ascomycetous yeasts (Richards et al. 2011). From mangrove swamps, vast numbers of marine fungal species are identified. Fungi that infect green algae such as *Rhizophydium littoreum* belong to marine chytrid (which is a primordial fungus). Lichens are complex microbes that arise from algae or cyanobacteria living among filaments of multiple fungi in a

symbiotic relationship, and generally ascomycetes or basidiomycetes were part of lichens. A number of lichens, such as *Pharcidia laminariicola*, *Turgidosculum ulvae*, *Arthopyrenia halodytes*, and *Pharcidia rhachiana* are observed in maritime ecosystems. *Aspergillus fumigatus*, *Candida albicans*, and *Saksenaea vasiformis* infect killer whales. A wealthy collection of fungal diversity is hitherto to be revealed and explored in the near future.

5.2.8 Marine Viruses

Viral particle is a tiny contagious agent that multiplies merely within the living beings and can transmit a disease to every type of life forms ranging from microbes such as bacteria and archaea to higher plants and animals (Koonin et al. 2006) and are present in more or less all ecosystem on our globe and are the most abundant variety of biological unit (Edwards and Rohwer 2005; Lawrence et al. 2009). Viruses are smallest among all living organisms, with size ranging from 0.02 to 0.2 μm, but some viruses may be up to the size of 0.45 μm (*Baculoviridae*) to 1 μm (*Paramyxoviridae*) which infect Crustacea and mammals, respectively. Marine viruses are the largest part among all biological entities in the ocean and exist in all places. The anticipated virus abundance in the marine ecosystem is 10^{30}, and each second around 10^{23} viral infections occur in the ocean and cause diseases and death of the life forms ranging from smallest prokaryotes to largest animals such as sharks or whales. Every viral attack potentially introduces novel hereditary material (DNA/RNA) into the host or into descendant viral particles, which results in the development of the host and virus; as a result, viruses are our globe's huge pool of genetic diversity. Till now microbiologists discovered ~5000 viruses, but our knowledge of the influence of viruses on global ecosystems is still very minimal.

Viruses comprise hereditary material such as DNA or RNA, enclosed by a protein coat. In some cases which is again surrounded by lipid bilayer. Viruses can only able to multiply in the host organism by taking control of host transcription and translational machinery and hence they are called as "obligate intracellular parasites." Every type of cellular life is vulnerable to viral attack, which shows that all forms of marine biota (such as algae, archaea, bacteria, fungi, invertebrates, plants, and vertebrates) are host to at least a single kind of virus. Although grazing by protists is a very significant part in microbial loop, viral lysis is also a very important type of transience imposed upon marine microbes. Viral biomass in the ocean water is substantial, and as viral number follows host abundance, bacteriophages are probably more abundant among viral community. Phytophages can stop phytoplankton blooms from growing still (Suttle 2005) and can able to control detrimental algal blooms along the coast (Lawrence and Suttle 2004). Genetic material such as DNA or RNA of viruses can either be double- or single-stranded, circular or linear. Single-stranded genetic material can be designated as +ve (which are similar like the ssDNA genome in which thymine replace uracil (like messenger RNA)) or -ve (complementary to the mRNA) sense genetic material. Viruses, one of the most swiftly developing genetic materials among every life forms, and the changes in their genetic

material are noncyclic. There were several viral families in the marine ecosystem which can cause diseases in diverse marine biota such as *Baculoviridae*, *Bunyaviridae*, *Coronaviridae*, *Dicistroviridae*, *Nimaviridae*, and *Parvoviridae* (crustacea); *Herpesviridae* (mollusks, fish, mammals); *Iridoviridae* and *Birnaviridae* (mollusks, fish); *Papovaviridae* (mollusks); *Reoviridae* (crustacea, mollusks, fish); *Caliciviridae* (fish, mammals); *Nodaviridae*, *Orthomyxoviridae*, and *Rhabdoviridae* (fish); *Paramyxoviridae* (mammals); *Picornaviridae* (algae, crustacea, thraustochytrids); *Marnaviridae* and *Phycodnaviridae* (algae); *Lipothrixviridae* (archaea); and *Corticoviridae*, *Cystoviridae*, *Leviviridae*, *Microviridae*, *Myoviridae*, *Podoviridae*, *Siphoviridae*, and *Tectiviridae* (bacteria) (Subbiah 2015). Viral infections put a large impact on hydroponics and marine microbes, and in few cases environmental stress or high abundance of culturable microbes permits stress to the organisms; as a result, viruses cause infections. Due to the advent of advanced molecular biology techniques, it becomes easy to detect many viral pathogens of marine organisms, and these methods will give quick information to prevent the expansion of viral infections to other members with the help of investigation at the prior phase of disease in fish farm hydroponic systems. Viral diseases can be controlled using recent advanced studies such as virus-host interactions and RNAi. Apart from scientific advancements, a small part of viral community only identified so far but their diversity is as vast as oceans and also as diverse as different microbial taxa are yet to be discovered.

5.3 Role of Marine Microbes

The distribution of marine microorganisms covers all corners of the ocean habitats right from top micro layers of the oceanic surface water to the abyssal depths (several kilometers from surface), and they flourish in all diverse environmental conditions where other higher organisms were not able to grow, such as warm waters (boiling) of hydrothermal vents to great chilled brine channels. Apart from their wide distribution, they are highly abundant on the planet Earth, and at the same time, they are functionally very diverse and take part in several diverse metabolic processes and are involved in almost every biogeochemical reactions taking place in the oceans. Some of the important metabolic processes are primary production (photosynthesis), photoheterotrophic, chemolithoautotrophic, chemoheterotrophic, grazing, lytic or lysogeny, diazotrophy, nitrification, denitrification, and decomposition.

5.3.1 Primary Production

Primary production is the manufacture of organic substances from aqueous or atmospheric CO_2; utilizing sunlight as energy source is known as photosynthesis, and most of the life on our globe depends either directly or indirectly on this important process, and the biota doing this are called as autotrophs or primary

producers, and are base of marine food web. In land ecosystems, this process is done mainly by plants while in aquatic ecosystems algae/phytoplankton. Cyanobacteria and eukaryotic phytoplankton (micro, nano, and pico) carry out photosynthesis in marine ecosystem by using sunlight as energy source to produce composite organic matter from inorganic molecules such as CO_2 and water (H_2O). Simplified representation of photosynthesis is as follows:

$$CO_2 + H_2O + \text{light} \rightarrow CH_2O + O_2$$

During this process compounds like glucose or other sugars (CH_2O) were generated which are polymer of reduced carbohydrates, which are base point from which all other complex molecules were synthesized such as composite carbohydrates, lipids, nucleic acids, and proteins. Heterotrophic organisms depend on these and transfer the organic carbon generated during primary production to higher trophic levels in the food web, fueling all of our globe's existing arrangement. Unlike terrestrial ecosystems, the preponderance part of photosynthesis in the marine ecosystem is carried out by phytoplankton (free-living microorganisms), and bigger autotrophs, for instance, the macroalgae (seaweeds) and sea grasses, are usually constrained to the littoral zone and adjoining superficial waters and attach to the bottom substratum. Major part of primary production takes place within microscopic fraction only. Among the phytoplankton community, eubacteria comprises significant proportion of primary producers in both marine- and land-based environments, and a few phototrophic archaea (were not part of oxygen production) (Schäfer et al. 1999) and several eukaryotic members are noteworthy supplier to the carbon fixation in the marine ecosystem, for instance, brown algae, green algae, and red algae and various types of single-celled microbes. Compared to land-based photosynthesis, oceanic primary production is limited by different and diverse factors. The sunlight and mineral nutrients play a vital function in controlling carbon fixation in the marine photic depths (Sigman and Hain 2012). As base of the food web occupies with primary producers in most of the habitats and without which other biota cannot sustain, they use the carbon produced by primary producers and respire the organic carbon using the oxygen generated by primary producers. Based on evolutionary history, the first organism on our globe is primary producers which are from ocean habitat. The primary producers are part of the carbon cycle and are in the starting point of the C cycle and take part in the biological pump. Primary producers are the only microbes in the ocean, which can create their own food from inorganic compounds, and without which there is no food for heterotrophs (bacteria, zooplankton, and even fish) to live.

5.3.2 Photoheterotrophy

Photoheterotrophy is a metabolic process where sunlight (energy source) is used, together with organic compounds (as their carbon source). The function of sunlight in the metabolic processes of bacteria in the marine ecosystems is hard to study,

although our knowledge is enhancing and carbon fixation by phytoplankton is only component of the whole representation. It is hard to distinguish photophosphorylation from oxidative phosphorylation and additional sort of ATP production. Instead of ATP generation, sunlight is used to create a proton-motive force, which can be utilized for the movement or for transportation of compounds into cells. Apart from this photoheterotrophic microbes might actively control ingestion and can be able to utilize sunlight for diverse metabolic purposes based on the energy needs of the microbe. In the oceanic ecosystem, this was carried out by three different groups of microbes, such as cyanobacteria (*Prochlorococcus* and *Synechococcus*), aerobic anoxygenic phototrophic (AAnP) bacteria, and proteorhodopsin-containing bacteria. Photoheterotrophic metabolism is widespread among numerically prevailing oceanic prokaryotes. Cyanobacteria such as *Prochlorococcus* and *Synechococcus* are able to uptake urea, DMSP, nucleosides, and amino acids by effective and competitive means (Michelou et al. 2007; Zubkov et al. 2003), at little native concentrations (Zubkov and Tarran 2005) for protein production. An approach of dynamic acquirement of a few organic substances by photoheterotrophic microbes is energetically additional advantageous over the de novo synthesis of the organic compounds. In this, both cyanobacteria compete with heterotrophs in nutrient-limited marine ecosystems. Shiba et al. (1991) discovered the AAnP bacteria from marine ecosystems. AAnP bacteria need O_2 for respiration and synthesis of photosynthetic units, and they comprise huge concentrations of carotenoid pigments compared to photosynthetic units (Rathgeber et al. 2004; Yurkov and Beatty 1998). Earlier studies show that their abundance is ~20% among bacterioplankton in the oceanic ecosystems. Schwalback and Furman (2005) noticed that AAnP bacterial abundance is only ~2.2% in the oceanic picoplankton community with the help of real-time polymerase chain reaction (RT-PCR) and infrared epifluorescence microscopy. In dilute and substrate-limited ecosystems, AAnP are proficient in cycling DOM and key elements and provide competitive gain to these microbes. Recent experimental data showed that in cyanobacteria and SAR11 alphaproteobacterium, organic substrate uptake is enhanced by sunlight (Mary et al. 2006). Surprisingly during recent environmental genomics surveys, proteorhodopsins (integral membrane proteins with retinal-binding) were discovered (de la Torre et al. 2003; Beja et al. 2000; Venter et al. 2004). The proteorhodopsins and bacteriorhodopsins act as sunlight-induced proton pumps and proficient in producing a chemiosmotic membrane potential from solar radiation and can be used for ATP production or to coerce active transport. Based on recent studies using genomic analysis, no proof of proteorhodopsin sunlight-induced metabolic processes could lead to photosynthetic carbon fixation, and based on those evidences, proteorhodopsin is able to merely sustain a photoheterotrophic growth. Aerobic anoxygenic phototrophic and proteorhodopsin-harboring bacteria are assumed to be copious and imperative machinery of the nutrient-depleted marine bacterial assemblages, as their latent exploitation of DOM and solar radiation would provide them a gain above their organotrophic counterparts.

5.3.3 Chemoautotrophy

Chemoautotrophy is a metabolic process in which microbes synthesize all required organic molecules by fixing CO_2 and deriving energy from chemical reactions. Chemosynthetic carbon fixation is common in the oceanic ecosystems; chemolithoautotrophic microbes utilize oxidation of reduced substances to produce ATP (energy) for CO_2 fixation. Earlier studies did not highlight these processes, but studies showed that localized chemolithoautotrophy is more significant. Chemoautotrophs make use of inorganic compounds as energy source such as H_2S, S, Fe^{2+}, H_2, and NH_3, and majority members are extremophiles that survive in intimidating situations (deep sea vents) and fix carbon in such environments, and they belong to diverse groups, for instance, anammox bacteria, ferrous iron oxidizers, halophiles, hydrogen sulfide oxidizers, sulfur oxidizers and reducers, hydrogen oxidizers, manganese oxidizers, methanogens, nitrifiers, and thermoacidophiles. Some of the chemolithotrophs grow much faster, for instance, *Hydrogenovibrio crunogenus* multiply in 1 hour (Dobrinski 2005; Boden et al. 2017). Based on recent isotopic studies, natural abundance of C^{14} and assimilation of $^{13}CO_2$ into membrane lipids of archaea presented primary evidences that oceanic archaea are autotrophs and part of primary production (Wuchter et al. 2003; Pearson et al. 2001). Herndl et al. (2005) showed that archaea can fix $^{14}CO_2$. Ingalls et al. (2006) showed that chemoautotrophy is a major contributor (>80% of archaeal C is from CO_2 fixation). Still there is very little knowledge available regarding chemolithoautotrophy and a lot of different microbial assemblages and biological processes in the deep ocean waters. Global estimate of chemoautotrophy is based on chemical energy released upon mineralization of organic matter. Anammox, hydrogen sulfide oxidizers, sulfur oxidizers, methanogens, and nitrifiers are abundant in oceanic anoxic or oxygen minimum zones and in sediments. The abundance of Thaumarchaeota members is considerable and oxidizes ammonium and can fix CO_2. There are some unique marine habitats where life is succeeded with the help of chemolithoautotrophy by oxidizing methane, sulfide, or alternative reduced elements (e.g., manganese and iron) such as black smokers, cold and hot seeps, and oxygen minimum zones (OMZs) in which life existence is independent of light and organic matter, which are unique to marine ecosystems.

5.3.4 Chemoheterotrophy

Chemoheterotrophy is the process where organic matter is utilized for their own metabolic processes and cannot fix CO_2. There are two types of chemoheterotrophs: chemolithoheterotrophs, which make use of inorganic compounds as energy resources, for example, sulfur, and chemoorganoheterotrophic microbes, which make use of organic compounds for ATP generation such as carbohydrates, proteins, and lipids (Dworkin 2006).

5.3.4.1 Chemolithoheterotrophy

Chemolithoheterotrophs gain ATP from the inorganic molecule oxidation but are unable to fix CO_2. The knowledge of chemolithoheterotrophic bacteria, which can oxidize reduced sulfur compounds, has been noticed to be more prevalent than formerly thought (Kuenen 1989; Sorokin 1991; Kelly and Harrison 1989). There are several bacteria showing this metabolism either facultative, such as *Oceanithermus profundus* (Miroshnichenko et al. 2003) and *Sulfitobacter pontiacus* (Sorokin et al. 2005), or obligate, such as *Catenococcus thiocyclus* (Sorokin 1992), *Desulfovibrio* (Kuever et al. 2005a), *Desulfobacterales* (Kuever et al. 2005b), and *Desulfuromonales* (Kuever et al. 2005c) and some species of the genera *Thiobacillus*, *Beggiatoa*, *Nitrobacter*, and *Hydrogenovibrio*, and these oxidize thiosulfate, sulfate, sulfur, nitrite, and hydrogen and use organic compounds as electron donors.

5.3.4.2 Chemoorganoheterotrophy

Chemoorganoheterotrophy is a metabolic process by which organisms gain ATP, organic C, and reducing equivalents for several metabolic processes from organic compounds. Majority of the bacteria are chemoorganoheterotrophs, e.g., *Actinobacteria*, *Bacillus* spp., *Escherichia coli*, etc. Other than that animals and fungus are too examples of chemoorganoheterotrophs. Fungi and animals are classified as heterotrophs by absorption and ingestion, respectively. Chemoorganoheterotrophs use organic C (e.g., glucose) as their C, ATP, and electron resources. In marine food web, chemoheterotrophs serve as consumers by breaking intricate organic molecules (carbohydrates, fats, nucleic acids, and proteins) synthesized by phytoplankton into small molecules (such as amino acids from proteins, fatty acids and glycerol from fats, glucose from carbohydrates and nucleotide, phosphate and sugar from nucleic acids) and liberate ATP by organic carbon oxidation and H_2 to CO_2 and H_2O, respectively. Catabolic reactions of organic substances are via fermentation or respiration. Chemoorganoheterotrophs metabolize by respiratory mode, results in ATP generation is linked with oxidative phosphorylation, and results in the liberation of oxidized C substances such as carbon dioxide and reduced substances like water, hydrogen sulfide and nitrous oxide into the air and Chemoorganoheterotrophy make up a huge part of the liberation of Carbon dioxide into the surrounding environment, making it accessible for phytoplankton as a source of C. In several cases, respiration results in the release of minerals. Nitrogen and sulfur in organic C resources are converted to NH_4^+ and H_2S by the processes named as deamination and desulfurylation, respectively, which are significant components of the N and S cycle. Hydrogen sulfide released during desulfurylation is again utilized by lithotrophs and phototrophs, while ammonium released during deamination is again utilized by lithotrophs to the forms accessible to phytoplankton. Corals are symbiotic relationships with autotrophic microbes and gain C in this fashion. In the marine ecosystem, all zooplankton are chemoorganoheterotrophs. Although yeasts and fungi use dissolved organic matter (DOM) or particulate organic matter (POM), and contend with archaea and

organotrophic bacteria in the marine ecosystem and the little wealth of yeasts and fungi in marine ecosystem point out that they were not competitive, due to their larger size and higher surface area vs volume ratios over archaea and bacteria, place them at a drawback in utilizing DOC.

5.3.5 Viral Role in Biogeochemical Cycles

Most abundant and diverse organisms of our globe are viruses, every living organism is at least host to one virus, and knowing evolutionary relationships is of significance in marine ecology. By lysing ~30–50% of microbial biomass every day, they contribute enormously to the biogeochemical cycles of the ocean, and as a result of lysis, carbon and nutrients are released into a marine ecosystem which are ultimately taken up by several bacteria and archaea for their growth and metabolic processes. Viruses influence the number and diversity of the host and contribute to genetic material exchange, which is a very significant part of the overall evolution of the host and viral particles. In the recent past, metagenomic analysis uncovered the viral diversity, which results in unraveling the significance of viruses in marine ecosystems. Viruses encompass a firm and mutualistic association with the host, and photosynthetic apparatus was noticed in the viral genome which may be useful for viral infection. In the viral genomes, there are several genes which are not known for any specific function and might participate in the virus-host relationship. Viruses control the populace of all diverse living organisms, liberate DOC, and intercede genetic material exchange between microbes.

5.3.6 Nitrogen Fixation (Diazotrophy)

Nitrogen is a very important element and used for primary production in marine ecosystems. As a result of the extremely steady triple bond within 2 N atoms, N_2 is more or less static and unavailable to most of the marine biota although it is a highly abundant gas in our environment. Very few bacteria and archaea can be able to reduce dinitrogen to NH_3 and assimilate it. The enzyme accountable for this process is nitrogenase and functions under anaerobic conditions, limiting diazotrophy in anaerobic ecosystems or in microbes which create low O_2 conditions for nitrogenase enzyme to function. Cyanobacteria are the major diazotrophs and are accountable for ~50% worldwide dinitrogen fixation. To protect nitrogenase enzyme from O_2, a feature called heterocyst was evolved in cyanobacteria, but *Trichodesmium* spp. a non-heterocystous cyanobacterium mainly distributed in tropical oceanic waters is known for aerobic nitrogen fixation. Some *Synechococcus* spp. were diazotrophic and were widely distributed in the marine ecosystems. Apart from this other organisms such as unicellular cyanobacteria UCYN-A and *Crocosphaera watsonii*, *Nodularia* spp. and *Anabaena* spp. (free-living heterocystous species), and the heterocystous diatom symbiont *Richelia intracellularis* are also part of marine nitrogen fixation, and most of them are primary producers. Fe and P are two

important micronutrients, based on the concentration in the oceanic waters effect the distribution and diversity of N_2-fixing microbes in the marine ecosystems, like high abundance of *Trichodesmium* spp., might be due to high concentration of Fe (coming from dust) in the North Atlantic Ocean and the Arabian Sea, but unicellular nitrogen-fixing cyanobacteria dominate in North and South Pacific Ocean and South Atlantic Ocean. Several chemoheterotrophic bacteria can also fix nitrogen but are considered to be uncommon in the marine ecosystems, as the provision of utilizable dissolved organic matter is very less to sustain the lofty budget of diazotrophy. Diazotrophy is not found in Eukarya but widely distributed in genetically, physiologically, and ecologically diverse archaea and bacteria (Young 1992), and nitrogenase genes are not conserved as the closely related species of the genus *Vibrio*, such as *Vibrio diazotrophicus*, can convert N_2 into ammonia but *V. cholera* cannot. Diazotrophy is common in metabolically diverse marine organisms such as in photolithotrophs (cyanobacteria, with heterocystous filamentous (*Calothrix*, *Aphanizomenon*, and *Nodularia*), some non-heterocystous filamentous (*Lyngbya*, *Oscillatoria*, and *Trichodesmium*), and unicellular (*Synechococcus* and *Gloeothece*) genera and in anoxygenic photoautotrophs (*Chlorobium*, and *Chromatium*), anoxygenic photoheterotrophs (*Rhodospirillum*), chemoheterotrophs (strict anaerobes (*Clostridium* and *Desulfovibrio*), aerobes or facultative (*Azotobacter*, *Klebsiella* and *Vibrio*)), and chemolithotrophs (archaeal methanogens and iron oxidizers (*Thiobacillus*)) (Table 5.2).

5.4 Potential Applications and Benefits

The impact of microbes from diverse marine habitats on human activity is unimaginable. They make the ecosystem function in a dynamic and healthy way such as regulating food webs, generation O_2, biogeochemical cycles, assuring ecosystem integrity, carbon dioxide uptake, and buffering. Apart from this marine microbes provide very significant value-added products to the mankind.

5.4.1 The Association Linking Biodiversity and Ecosystem Stability

In the coming years, climate alteration and anthropogenic ecosystem alteration will endure causing biodiversity loss (Sala et al. 2000), apart from the huge species extinction rates already noticed in the present-day world (Stork 2010). Biodiversity is the description of species diversity. Species contribute crucial functions in ecosystems. The stability of the ecosystem services on which humans depend will be threatened as a result of local and global species losses (McCann 2000). There could be numerous interactions among diversity and stability, depending on how we define stability (Ives and Carpenter 2007). An essential ecological belief is that association of linking biodiversity and ecological stability that an environment has a huge number of possible explanations to tribulations has more likelihood of extant ecosystem pressure. In some ecosystems, one microbe (newly emergent) will multiply faster (due favorable conditions) and replace dominant one, but in general

Table 5.2 Microbial functional groups in the marine ecosystem

S. no.	Functional group	Microbes	Role of microbes	Probable location
1.	Primary producers	Cyanobacteria, microalgae, and pico-eukaryotes	Inorganic carbon fixation to produce organic carbon using light energy	Photic zone (few centimeters to ~200 m)
2.	Photoheterotrophs	Cyanobacteria and other bacteria	Utilization of light energy along with organic material	Photic zone (few centimeters to ~200 m)
3.	Chemoautotrophs	Bacteria and archaea	Sulfate reduction, anammox, ammonium oxidation, hydrogen sulfide oxidation, sulfur oxidation and reduction, hydrogen oxidation, methanogenesis, ferrous iron oxidation, manganese oxidation	Anoxic zones, deep ocean vents
4.	Heterotrophs	Bacteria and archaea	Oxidize dissolved and particulate organic matter to generate biomass and minerals	Throughout ocean habitats
5.	Grazers	Protozoa, protists, nanoflagellates	Use organic matter of cyanobacteria, bacteria, and archaea, release dissolved organic matter	Throughout ocean habitats
6.	Lysis of host, generation of DOC	Viruses	Control the population of all life forms, release DOC, and mediate a genetic material exchange between microbes	Throughout ocean habitats
7.	Nitrogen fixers (diazotrophs)	Cyanobacteria, bacteria, and archaea	Reduction of nitrogen molecule to ammonium	Photic zone (few centimeters to ~200 m)
8.	Nitrifiers	Archaea and bacteria	NO_3 oxidation from NH_4^+	
9.	Denitrifiers	Archaea and bacteria	Release N_2 or N_2O gas	
10.	Decomposers	Fungi	Oxidize particulate organic matter to generate biomass	Water and sediment, parasites on other eukaryotes, and as symbionts (lichens, plants and algae)

Fig. 5.1 Potential applications of marine microbes

community recital and ecosystem functioning remains typically unaffected. This is called "insurance hypothesis" which is defined as follows: Biodiversity assures ecology beside turndown in its performance, so that several species afford superior assurances that a number of taxa will preserve functioning still if others not succeed. Preservation of biodiversity, together with microbial biodiversity, is crucial for ecological performance and the people welfare (Fig. 5.1).

5.4.2 Bioactive Compound Discovery

Oceanic environment is promising as a "gold mine" for discovery of novel bioactive substances. From diverse marine biota, such as algae, aplysia, ascidians, bryozoan, corals, fish species, microbes, sea cucumbers, sea hares, sponges, and worms, a huge

number (>20000) of structurally varied oceanic natural substances have been extracted (Blunt et al. 2012; Bharate et al. 2013) and present an attractive and exigent plan for creating novel units by means of synthetic chemistry. Marine microbes persist to be a key spotlight of numerous bioactive compounds research works, with around 10% augment in the quantity of substances reported between 2011 and 2012 (Blunt et al. 2012). Marine invertebrates and plants, specifically, embody an ecosystem wealthy in microbes which generate bioactive substances with unique characteristics, for instance, antibacterial, antifungal, antiviral, anticancer, antifouling, and antibiofilm activities. The main drawback of mining for novel bioactive compounds is their cultivability; as of now merely 1% of microbes can be cultured by means of conventional culturing methods.

5.4.2.1 Antibacterials

The appearance of multidrug-resistant bacteria coerces the incessant hunt for novel antibiotics. Since the decline in terrestrial antibiotic discovery, scientists are looking into diverse marine ecological niches for the same. There are several types of compounds with antibacterial activity isolated from the diverse ocean habitats such as alkaloids, polyketides, peptides, sterols, and terpenes. According to recent WHO notice, there are three groups of antibiotic-resistant bacterial (ARB) pathogens appearing as foremost intimidation to the human health: Priority1 (Critical), carbapenem-defiant *Acinetobacter baumannii*, *Enterobacteriaceae*, and *Pseudomonas aeruginosa*; Priority 2 (High), *Campylobacter* spp., *Enterococcus faecium*, *Helicobacter pylori*, *Neisseria gonorrhoeae*, *Salmonellae*, and *Staphylococcus aureus*; and Priority 3 (Medium): *Haemophilus influenzae*, *Shigella* spp., and *Streptococcus pneumonia*. Even though the imperative necessity for novel antibiotics is to predominantly deal with the increase of ARB, novel antibiotic discovery is progressing leisurely and there are not many substances in the pipeline. Apart from this several compounds which might potentially fight with pathogenic bacteria isolated from the oceanic environment, structurally novel substances, for example, abyssomicin C (Keller et al. 2007) and marinopyrrole A (Haste et al. 2011) with positive action toward methicillin-resistant *Staphylococcus aureus* (MRSA), the alkaloid cyclostellettamine F (de Oliveira et al. 2006) with activity against *P. aeruginosa*, and trichoderins (Pruksakorn et al. 2010), novel aminolipopeptides with anti-mycobacterial activity. Mayer et al. (2013) catalog 23 antibacterial compounds in preclinical pharmacological research. Jang et al. (2013) discovered a polyketide antibiotic (anthracimycin) which acts against *Bacillus anthracis*. Marine pharmacology reviews the antibacterial compounds and their potential activity against bacterial pathogens (Mayer et al. 2013, 2017).

5.4.2.2 Antifungals

Incessant hunt for novel antifungal agents is taking place for multidrug resistant fungi, such as *Aspergillus fumigatus* and *Candida albicans* strains in recent works. In contrast to infections by drug tolerant bacteria, resistant pathogenic fungi present quite rare. Nevertheless, 40% of patient's demise is due to hospital-acquired bloodstream diseases caused by *Candida* spp., while strewn infections due to *Aspergillus*

are able to kill ~80% of infected persons. Antifungal molecules of maritime source with activity toward these pathogenic fungi comprise the kahalalide F which is a cyclic depsipeptide (Sparidans et al. 2001). Several secondary metabolites, with significant antifungal properties, were extracted from oceanic microbes, for instance, algae and invertebrates, in between 2000 and 2015; approximately 65% of oceanic natural substances comprising antifungal properties were extracted from bacteria and sponges (El-Hossary et al. 2017). Lacret et al. (2015) isolated novel ikarugamycin derivatives with antifungal activity from *Streptomyces zhaozhuensis*. Marine pharmacology reviews the antifungal compounds and their potential activity against fungal pathogens (Mayer et al. 2013, 2017).

5.4.2.3 Antivirals

Viral diseases such as AIDS and influenza caused by human immunodeficiency virus and influenza A subtype (H1N1) are the main hazard to public health, and rapid evolution and development of resistant viruses to antiviral drugs, isolating novel antiviral substances is of top significance. A wide variety of antiviral molecules were extracted from the oceanic environment such as cyclic alkaloids, depsipeptides, macrolides, nucleosides, polysaccharides, and terpenes. The antiviral activity of adenine arabinoside was originally studied by Privat de Garilhe and De Rudder (1964) which is the most significant antiviral drug from marine ecosystem and is named as vidarabine (nucleoside Ara-A) which is extracted from *Tethya crypta* (sponge) and is produced with the help of the structure of nucleosides spongouridine and spongothymidine and inhibits herpes, varicella zoster, and vaccinia viruses by acting on viral DNA polymerase and DNA synthesis (Sagar et al. 2010; Mayer et al. 2010). Mycalamide A and B isolated from *Mycale* (sponge) inhibit the polio type I and herpes simplex type I viruses at a level of 1–2 and 5 ng/disc (Perry et al. 1990) and inhibits translation (RNA to proteins) (Burres and Clement 1989). Avarol, a hydroquinone (sesquiterpenoid) with a reshuffled drimane skeleton, extracted from the *Dysidea avara* (a marine sponge) showed inhibition on the multiplication of AIDS virus and T-lymphotropic retrovirus (HTLV III) at a quantity of 0.1 μg per mL and the mechanism involved in blocking viral replication (Sagar et al. 2010) and a sum of forty antiviral molecules, formally permitted for medical management of different viral diseases and about 50% for the HIV treatment (Sagar et al. 2010). Some of them are papuamides A, B, C, and D and microspinosamide (cyclic depsipeptides), 4-methylaaptamine, dragmacidin F and manzamine A (alkaloid), hamigeran B (phenolic macrolides), and cyanovirin-N (protein) (Sagar et al. 2010; Zappe et al. 2008).

5.4.2.4 Anticancer Compounds

Majority of bioactive molecules derived from marine ecosystem comprises anticancer properties. But, fairly a small number of compounds go through preclinical and clinical examinations and merely a minute group from microorganisms. Cytarabine (Ara-C) is chemically synthesized using nucleosides from spongouridine and spongothymidine, formerly extracted from the *Tethya crypta* (marine sponge), and used as medicine for over 40 years for the treatment of acute leukemias and some

lymphomas. The US Food and Drug Administration (USFDA) has permitted Yondelis (trabectedin) to treat liposarcoma and leiomyosarcoma that have extended to additional parts of the body and/or cannot be aloof with surgery developed by Pharma Mar from molecules released by the *Ecteinascidia turbinata* (tunicate). Antiviral drug with antiproliferative action by jamming the cell division and apoptosis induction process, with brawny action on multiple myeloma cells extracted from *Aplidium albicans* (Mediterranean tunicate), is aplidine (dehydrodidemnin B), a depsipeptide dehydrodidemnin, and is developing for the treatment of multiple myeloma (stage 3 of experimental tests), and for hematological malignant neoplasias and solid, like T-cell lymphoma (stage 2 of experimental tests) (Jaspars et al. 2016). A marine anticancer compound from *Aspergillus* sp. (marine fungus) that restrains polymerization of tubulin results in the distraction of the vascular endothelial architecture of a tumor by plinabulin gone to phase III clinical trials. Salinosporamide A (marizomib) is a new, powerful proteasome inhibitor isolated from the *Salinispora tropica* (marine actinomycete), stimulates apoptosis by means of caspase-8-reliant mechanism in leukemia and multiple myeloma cells (Potts et al. 2011). The tetrodotoxin (which can block voltage reliant Na^+ channels) extracted from algae, bacteria, and fish has revealed an analgesic property in sarcoma patients (Potts et al. 2011).

5.4.2.5 Antifouling Properties

Biofouling, the objectionable accretion of microbes such as algae, plants, and/or animals on dampy substrata, is of huge disquiet in a broad variety of applications, ranging from food packaging/storage, industrial equipment, and marine vessels to medical devices and water purification systems. To fight against biofouling, two important strategies are used either avert preliminary attachment or to demean fouling biofilms. Earlier organotins were used as an effective antifouling coating, but due to their toxic effect on marine biota, it urges the use of non-toxic novel biofouling compounds. Marine invertebrates and seaweeds have established to be a flourishing resource of antifouling molecules, but it is hard to get them in hefty amount. To combat these difficulties, marine microbes were studied to discover new antifouling compounds which can be synthesized in huge concentrations. In this context, a number of fatty acids such as 1-hydroxy myristic acid, 9-Z-oleic acid, and 12-methyl myristic acid have promising antifouling properties isolated from marine microbes. Synthetic alkyl butenolide, following alkylated butenolides obtained from an unfathomable sea *Streptomyces* species, was established to show potential antifouling properties which can combat attachment of micro- and macro-fouling biota for several months together like coatings of 10% fatty acids prevented the same for 1.5 year. Apart from the above, a number of compounds such as 2-alkylquinol-4-ones, phenazine-1-carboxylic acid, and pyolipic acid extracted from a *Pseudomonas* sp. demonstrate strong antifouling activities (Eguia and Trueba 2007; Burgess et al. 2003). Satheesh et al. (2016) reviewed the potential of several natural antifouling molecules synthesized by marine microbes which is a valuable information.

5.4.2.6 Antibiofilm Properties

Quorum sensing is a communication mechanism between two cells which harmonize genetic material expression in reaction to population cell density and depends on the release of tiny compounds, which once sensed in lofty amounts show the way to synchronized actions by amending few physiological processes such as biofilm formation, antibiotic resistance, bioluminescence, motility, and virulence factor production. Biofilm development is a supportive, collective action that involves bacterial cell existence entrenched in a self-generated extracellular matrix, and cells stick to one another or to an exterior part of huge disquiet for the treatment of bacterial diseases as biofilm-associated bacteria showing augmented profiles of antibiotic resistance in contrast to their sessile complements. Antiquorum sensing compounds frequently display antifouling/antibiofilm properties as both biofilm formation and quorum sensing are intimately associated. Papa et al. (2015) studied the anti-biofilm property of cell-free supernatants which are resultant from planktonic and sessile cultures of cold-adapted microbes belonging to *Psychrobacter*, *Pseudoalteromonas*, and *Psychromonas* species which were tested against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. EPS273 (exopolysaccharide) isolated from the marine bacterial culture (*Pseudomonas stutzeri*) supernatant 273 efficiently restrains biofilm development and disperses preformed biofilm of *Pseudomonas aeruginosa* (Wu et al. 2016) which is the key reason of morbidity (unhealthy state) and mortality amid patients with cystic fibrosis disease. Biofilm inhibitors and quorum sensing might offer new treatment alternative for treating bacterial infections and are immediately required owing to the appearance of several MDR bacteria.

5.4.2.7 Marine Enzymes

Marine ecosystem, which covers ~70% of our planet exterior and an enormous reserve of valuable enzymes, stays less explored. Microbes mineralize intricate organic matter (OM) with the help of diverse pathways of degradation with their metabolism in the oceanic ecosystem and add to the secondary production in the ocean. Marine microbes in the utilization of food often use cell-free extracellular enzymes. With huge biochemical and genetic diversity, aquatic microbes are of attention as a potential resource of enzymes with inimitable properties, for instance, barophilicity, cold adaptation, hyperthermostability, and salt tolerance. Marine bacteria and fungi exude diverse enzymes with respect to their environment and their ecological function. Global enzyme market of proteases account for >60% (Zhang and Kim 2010) and are of the most broadly studied enzymes from ocean and are immensely utilized in the detergent, in biotechnological research, food processing, leather and pharmaceutical industries and have latent functions in waste management and bioremediation. Both neutral and alkaline proteolytic enzymes are isolated from different species of *Bacillus* and acid proteases obtained from fungi. Marine proteases extracted from extremophiles are quite stable in the presence of a broad variety of chemicals and comprise most of the desired properties for diverse biotechnological applications (Chellappan et al. 2011; Kumar et al. 2004; Sana et al. 2006). Apart from proteases, marine microbes also release other extracellular enzymes such as lipases and

polysaccharide-degrading enzymes and later enzymes useful in bioethanol production, as a result of green energy endeavors (promising clean alternative to fossil fuels) which is urgently needed. Recent studies concentrated on the enzymes which can convert the biomass of marine cyanobacteria into ethanol will reduce the impact of food shortage as a result of bioethanol from crops. Oceanic enzymes have significant role in the bioremediation of polluted waters (Sana 2015). Several enzymes with diverse functions are isolated from oceanic microbes such as agarase, alginate lyase, α-amylase, cellulases, chitinase, chitobiose-deacetylase, cyclomaltodextrin-glucanotransferase, β-fucosidase, α-glucosidase, α-galactosidase, β-galactoside-α-2, β-galactosidase, N-acetyl glucosamine-deacetylase, N-acetyl glucosamine-6-phosphate-deacetylase, β-glucosidase, endo-1,3-β-D-glucanase (I), endo-1,6-β-D-glucanase, pullulanase, κ-carrageenanase, 6-sialyltransferase, endo-1,4-β-xylanase, lignin-modifying enzymes (LMEs): manganese-dependent peroxidase (MnP), lignin peroxidase (LiP), laccase, catechol-oxidase, cresolase (monophenol monooxygenase), alkaline protease, L-asparaginase, collagenase, L-glutaminase, β-1,4-mannanases, poly-β-hydroxyalkanoate (PHA)-depolymerase, poly(3-hydroxybutyrate)-depolymerase, lipase, hydrogenases, metal neutral proteinases, neutral protease, subtilisin (EC-3.4.21.62)-like serine protease, thiol protease, tyrosinase, superoxide-dismutase, glucose-dehydrogenase, etc. (Chandrasekaran and Rajeev Kumar 2010).

5.4.2.8 Marine Nutraceuticals

Change in way of life builds the hazard for a few diseases, for example, cardiovascular illness, corpulence, diabetes, and cancer in the ongoing past have tossed an unswerving test to our food habits. Nutraceuticals or functional foods are noticed and highly recommended as the methods for the above disease prevention. In the recent past, there is immense attention in marine nutraceuticals as they show positive effects on human health and have medicinal features (therefore the term -ceutical) and include active components, for instance, antioxidants, enzymes, essential oils, phytochemicals, and vitamins which are found to have uses as antioxidants, cofactors, and oils, as natural additives in foodstuffs, as dietary complements together with tint additives, and as vitamins, which augment human health, and among huge number of nutraceuticals, carotenoids, chitin oligosaccharides, collagen, fucoidan, glucosamine, omega-3 fatty acids, and mineral supplements are prevalently consumed. Chitin is the second most plentiful standard polymer after cellulose has tremendous industrial value because of its non-toxicity, biodegradability, hydrophobicity, and physiological latency. For instance, enzyme-immobilized chitin is utilized for clearing up of fruit juices and handling of milk in the food industry (Krajewska 2004). Although chitin and chitosan have important characteristics, their use is limited due to solubility, but the chitosan oligosaccharides (COS) [after enzymatic digestion of chitin or chitosan] are widely used as retaining the parent molecule properties along with water soluble property. COS shows potential activities against aging, allergy, asthma, cancer, diabetes, hypertension, inflammation, intracellular oxidative stress, microbial infections, photoaging, obesity, and osteoarthritis (Himaya and Kim 2015). An edible film is a promising approach in the food industry based on chitosan. Glucosamine is a sugar with

amino group and is a standout among the most rich monosaccharides and is a piece of human bone tissue and consequently is utilized as enhancement to alleviate from joint manifestations (Anderson et al. 2005), have lubricant characters and ligament building for the joints (Gorsline and Kaeding 2005) and furthermore has appeared provocative action by smothering inflammatory actuation of endothelial, intestinal epithelial and synovial cells (Nagaoka et al. 2011). Apart from using as a nutritional supplement, it is also used in beverages which become a more successful approach than beverages with cereal products (Barrow 2010). Sulfated polysaccharides (SPs) envelop a mind-boggling gathering of macromolecules which have lofty level of structural variety in light of difference in subatomic weight, disaccharide development and sulfation, and marine algae growth are prestigious asset of organically dynamic SPs, which can enhance the intrinsic safe response and consequently support the tumoricidal activities of macrophages and natural killer cells (Yim et al. 2005) and is in charge of the anticancer property of marine-sulfated polysaccharides, for example, chondroitin-4-sulfate, chondroitin-6-sulfate, fucoidan, heparin and pentosan polysulfate (Himaya and Kim 2015). The human body cannot be able to synthesize omega-3 fatty acids (polyunsaturated fatty acids (PUFAs)); this makes it an important supplement that must be taken to sustain the physiological activity of the body. Aside from this, few PUFA, for example, α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), have built up to hold tremendous scope of medical advantages, for example, counteractive action of arthritis, atherosclerosis, blood platelet aggregation, cystic fibrosis, coronary heart diseases, general inflammation, hypertension, hypertriglyceridemia, ocular diseases, several carcinomas, and type II diabetes (Lavie et al. 2009; Wu and Bechtel 2008; Mazza et al. 2007; Shahidi and Wanasundara 1998; Mullen et al. 2010). Although PUFA are mostly extracted from fish, fish even cannot synthesize the same but depend on marine microalgae which are a prosperous resource of PUFA with long chain where it represents for 10–20% of cell load in a few species (Himaya and Kim 2015). The advantage of PUFA generation from microalgae is further eco-manageable and less harming to the ecosystem. Carotenoids from marine microbes have high market value and have many prospects as feed supplements, nutraceuticals, food colorants and in favor of pharmaceutical and cosmetic uses and more than 600 known carotenoids, with astaxanthin, β-carotene, fucoxanthin, α-carotene, lutein, zeaxanthin and lycopene being the most well-known. Astaxanthin is an ideal fixing decision for antiaging, anti-fatigue, eye security, skin well-being, or any condition where assurance from free radicals is required (Fiedor and Burda 2014) and fucoxanthin used to treat the corpulence decrease (Gammone and D'Orazio 2015).

5.4.2.9 Marine Cosmeceuticals

The skin is a chief intent of beauty healings and the additional targets are hair and nails. Several cosmeceuticals are extracted from diverse marine habitats, and hunting for new molecules is becoming extensive in cosmeceutical industry. Exopolysaccharides (EPS) are most commonly utilized in the individual consideration bioactive fixings segment and various microorganisms produce EPS, including

archaea, cyanobacteria, and *Proteobacteria*. New healthy skin techniques point various maturing mechanisms by using functional dynamic ingredients in amalgamation with pioneering conveyance systems to build stratum corneum porousness for restorative plans and novel conveyance systems, for example, microcapsules, nanoparticles, and lipid systems (Golubovic-Liakopoulos et al. 2011). Balboa et al. (2015) reviewed broadly the scenario of the cosmetic industry with respect to marine sources, regulations used, targets and delivery systems, components, and major functions. Deepsane an aged concentrate containing the EPS HYD657 discharged by the strain *Alteromonas macleodii* subsp. *fijiensis* biovar *deepsane* (Cambon-Bonavita et al. 2002) and is industrially reachable under the name of Abyssine for reducing irritation and soothing of susceptible skin beside mechanical, chemical, and bright (UV B) hawkishness (Martins et al. 2014). An amazing antioxidant that represses harm from responsive oxygen species, secures common protection enzymes, shields from UV harm, and reestablishes obstruction lipids is a fermented product of the remote ocean hydrothermal vent bacterium *Thermus thermophilus* (Venuceane^{TM} delivered by Sederma Cosmetics). Microalgae are conventional on the market for face and healthy skin products, for example, regenerant creams, antiaging, anti-irritants, emollients, hair care products, invigorating items, and sun protection are commercially accessible (Spolaore et al. 2006), for example, Alguard (secures against photodamage, micro-abrasion of the skin, and aging), Alguronic Acid (huge antiaging characteristics, reviving the skin for an increasingly energetic appearance), Dermochlorella DG (builds immovability and skin tone), and XCELL-30 (follows up on cell turnover in the basal layer of the epidermis, in this way permitting the protection of the young qualities of the skin) (Jaspars et al. 2016).

5.4.3 Marine Bioremediation

Bioremediation is at present the best triumphant arrangement to mitigate and to recuperate places polluted with hydrocarbons and has been the perfect system for clean-up contamination around the seas. Oil-derived items are the main asset of power for industry and social orders. Major part of crude oil or petroleum products is transported enrooted sea through ships around the world, which is the major and frequent cause of significant oil spills in the marine ecosystem (Harayama et al. 1999). It is extensively well-known that petroleum hydrocarbon pollution has influenced and spoiled the marine habitats and represents a regular menace to the ecosystem health and sustainability (McGenity et al. 2012). The recent devastation and consequences' leftovers after roughly 0.6 million tons of raw petroleum was discharged by the Deepwater Horizon outburst in the Bay of Mexico have augmented the amount of oil products released into the oceanic ecosystem consistently (~1.3 million tons) (Crone and Tolstoy 2010). Bioremediation is considered as the major efficient with striking dispose of biodegradation biotechnology to diminish echelon of contamination as well as to recuperate polluted marine ecosystems (Kumar et al. 2011). The bioremediation depends on the exploitation of the various

metabolic potentials of microbes or their enzymes or other biomolecules such as biosurfactants for the degradation and elimination of several marine pollutants (Das and Chandran 2011; Medina-Bellver et al. 2005). It is widely utilized because of the expanded reasonable methodologies of natural attenuation and biodegradation, in most of regular marine living ecosystems (Kumar et al. 2011). Amid oil slicks the accompanying bioremediation approaches are typically connected in marine biological systems: (i) addition of hydrocarbon-degrading microbes to supplement or to increase the current microbial biota, which is called bio-augmentation, and (ii) addition of composts (supplements), to influence and stimulate the development of local hydrocarbon degraders that is named bio-stimulation (Das and Chandran 2011). However, additional investigations are needed to unearth the appropriate mixture of microbes and environmental characteristics to get better bioremediation processes. Indeed, for a remediation procedure to be successful, in general pace of degradation needs to be accelerated higher than existing microbial processes. A sorted out move to pick adept microbial species for a specific remediation purpose is fundamental to exploit the dissemination of the contaminant in the marine environment. *Pseudomonas chlororaphis* releases pyoverdin, which augments the organotin substance degradation in marine waters. A few biopolymers and biosurfactants are likewise used to ecological waste administration and treatment (Cohen 2002; Jones 1998; Milanese et al. 2003; Matsunaga et al. 1999; Watanabe 2001).

5.4.4 Food from Marine Fisheries and Aquaculture

The oceanic environment is the significant resource of quality food which can meet the global population demand which is ever increasing day by day. World fishery catch has reached its highest sustainable yield, but, if the present tendency of catch persists, several saleable stocks of fisheries might crumple by 2030. Recently aquaculture production has increased by over 10% every year and presently that represents over half of all shrimp/fish consumed; this is imperative in giving a manageable asset of marine nourishment later on. Marine organisms oversee the food web and are the foundation of the creation of the food for each living being in the sea. Naturally, microbes are of immense significance to the fisheries and aquaculture segment, and improved information of their function will be significant to make safe the essential food provisions from oceanic biota later on. Fish yield depends on the ample performance of microbes in the ecosystem. Hitherto, regardless of their vital function in marine food webs, our knowledge is very diminutive regarding oceanic microbial assemblages working and how changes, as a result of pollution or environmental change, may impact commercial fish stocks and these lacunas to be filled by the marine microbial research. Microbiological exploration has added to upgrading hydroponics growth systems. Microbial bioremediation of in land-based mariculture and microbial management of rigorous making frameworks have upgraded restriction and ecological rapport. An important area of applied research in commercial aquaculture sector is infections in cultured fish and shellfish stocks. Antibiotic usage is in dealing with bacterial infections creating multiple drug-resistant microbes, which ultimately

corner human health. Recent studies improve the knowledge of host-microbe interactions within limited marine habitats, which may understand disease progress overall and instructions to battle developing diseases in an increasingly natural manner, for example, the utilization of probiotics and immune stimulants, for example, yeasts for disease management through the preventive approach shows interesting results. Functional feed (FF) advancement as food with nutritional components that present growth, good health, and ecological and cost-effective profits ahead of conventional feeds corresponds to an immense prospect to ensure the eventual fate of hydroponics (Olmos et al. 2011). FF must be essentially enhanced with raised dimensions of vegetable protein, complex starches, and solely picked harmless probiotic microbes. Exploitation of probiotic microbes has risen as an answer with huge applications in the hydroponics business. *Bacillus* species are the majorly explored microbes for animal probiotic advancement because of the flexibility of their development supplements; grandiose echelon of enzyme release to enhance feed deterioration, upgrading its absorption; affecting positively on hosts by altering the host-related microbial network and immune system; discharge of antimicrobial substances and enhances the quality of ecological parameters (Olmos et al. 2011). *Bacillus subtilis* is commonly perceived as safe; it tends to be utilized as a FF in the hydroponics business. In a latest study of supplementation with the elevated quantity of soybean meal, the lofty quantity of composite starches and a probiotic strain (*Bacillus subtilis*) on *Atractoscion nobilis*, *Oreochromis niloticus*, and *Litopenaeus vannamei* resulted in excellent outcomes which open immense prospects to novel FF development for shrimp and fish. Enzymes from *Bacillus* are exceptionally productive in separating a huge assortment of proteins, starches, and lipids into basic units. *Bacillus* species multiply competently with very low-cost C and N sources (Sonnenschein et al. 1993) and degrade organic aggregated trash in lakes of shrimp cultures (Lin 1995; Rengpipat et al. 1998; Guo et al. 2009). Investigations in probiotics have primarily concerted on pathogen elimination, competition, or microbiota management. Infectious microbial control might effect in better disease supervision, in addition to having constructive outcomes on the ecosystem. Novel methodologies and strategies, for example, recirculating aquaculture systems (RAS) and integrated multi-trophic aquaculture (IMTA), are likewise ending up perpetually critical in the chase for extra-natural benevolent and capable production systems. In RAS, seafood advancement is pooled with water purging to safeguard a sound culture biological system, much of the time in an encased framework. RAS are exceptionally developed and unpredictable aquaculture frameworks that rely upon organic procedures which are chiefly microbial and can, in this manner, promote from advancement in marine microbial ecology. Furthermore, RAS open the chance to amalgamate microalgal frameworks into it and also downstream in the administration of fish handling outpourings. Biology of microbial networks in RAS and its connection with the microorganisms in the food and gut of cultured living beings is still deficiently known, and the organisms possess amid larval advancement that is exceedingly variable and is considered to induce larval viability and fitness. Effectively performing microbes are furthermore mandatory to guarantee that the cultured marine-foodstuff is well and to avert the propagation of superfluous

with infectious microbes such as several known microalgal species release strong toxins which, in the case of bloom, may impair oceanic living beings and make economic damage to hydroponics industry or on the other hand a restriction on offers of the items for human well-being contemplations.

5.4.5 Energy from Biofuels

Most common difficulties we face are the extended period provision of reasonable power. In the twenty-first century, the vital concern for human sustainability is measured in terms of energy. The world's reliance on untenable nonrenewable energy sources (roughly 90%) and the developing populace demand novel resources of energy for continuity of human functions. Marine living resources can add notably to safeguard the rising requirement for energy. Microbial-enhanced oil recovery focuses on utilizing marine microorganisms to progress the resurgence rates of traditional fossil oil holds, which is finished by either diminishing the thickness of oil or the penetrability of the stone material in which the oil exists in. Another focus for sustainable fuel source using microbes is the preparation of biofuels using microalgae. The above applications entail an exhaustive acquaintance of marine microbial diversity and their usefulness in order to choose potent microbes and/or to recognize by-products. Sustainable biofuel production ensures eco-friendly processes and respective associations with different fields, such as the decrease of the greenhouse gas emission, and can be accomplished without effecting air, water, soil, or biodiversity and so forth. These problems can be solved by using microalgae; nonetheless, social acceptability and monetary reasonability still should be survived (Singh et al. 2012). Microalgae are capable renewable feedstocks for the production of biofuels and biorefineries. Microalgae can be cultivated in sewage, manure, and salt water and do not need productive soil and requires minimum energy for processing (Singh and Ohlsen 2011). Microalgae are documented as quickly growing photosynthetic microbes and have been accounted for to achieve transformation of 5–6% of approaching light energy into biomass. It is acknowledged that can transfer different feedstocks for biodiesel because of their hoisted vegetable oil content and biomass production rates. In microalgae the lipid content may be triggered (from 15.3% to 41.1%) by the nitrogen depletion (Ho et al. 2014). Based on these some researchers pointed out that microalgae may soon become a vital renewable fuel resource (Gouveia and Oliveira 2009). The major benefit of microalgae species is the lofty content of oils in the cytosols and chloroplasts. Among major crops which are currently used for biodiesel production microalgae yields highest (100000 L/ha) compared to palm (5950 L/ha), *Jatropha* (1892 L/ha), and canola (1190 L/ha) (Paniagua-Michel et al. 2015; Chisti 2008). The promising and clear capability of microalgal biofuels for contributing to environmental, social, and economic sustainability require to be changed into a practical reality.

5.4.6 Concluding Remarks

In the marine ecosystem, around 90% of the biomass is microbial and characterize a huge living diversity. A small number of microbes dominate and direct the most biogeochemical cycles in the ocean. Immeasurable microbes are rare but stand for a nearly unlimited genetic pool from which the marine microbes pull-in in order to react to ecological changes, thus maintaining the steadiness of the ecosystem. Seawater comprises vast diversity from prokaryotes such as bacteria and archaea and eukaryotes such as microalgae, protists, zooplankton, and several other higher biota and also includes an enormous quantity of viruses. Viruses manage biomass of microorganisms by "killing the winner" and create and preserve biodiversity as they are the major intermediaries of genetic exchange among microbes. The dynamics of each and every ocean biome is controlled by the stability of marine biogeochemical cycles and their compounds. Consequently, gaining knowledge about the ecology of marine microbial diversity is indispensable for the understanding of marine ecosystem, for the better and sustainable use of the ocean and to be able to foresee changes in ecosystem functioning. Fishing is the chief human activity as it supports a most vital ingredient (protein) supply for people. Fish production depends normally on primary production by phototrophic microbes, which, thusly, relies upon the availability of nutrients. Excess nutrients may lead to the development of HABs and few species produce toxic chemical and may cause serious health problems to local biota and also human. Immense nonpoisonous algal blooms may diminish water quality and cause anoxia. Natural carbon synthesized by phytoplankton and its transmission through the food web results in a huge amount of dissolved organic carbon (DOC) which is degraded or mineralized with the help of microbial food web, reusing nutrients which offer ascent to new biomass. The viral circle accelerates the recycling of carbon and nutrients. Microbial food web is responsible for the biological pump, namely, the transport of CO_2 as organic carbon to the deep sea and sea floor. The organization and utilization of marine microbes has a vital function in sustainable growth with the industrial and profitable application of microbial diversity value millions of pounds. Given the heterogeneity of marine ecosystem and the huge significance of microbes to offer new pharmaceuticals, chemicals, and novel technologies, the biotechnology industry has an enormous, principally unexploited reserve for the unearthing of new biomolecules and novel processes. Although marine microbes has significant role in sustainable development, very little is recognized regarding the significant role of microbial diversity to national economy, to prosperity creation and in the eminence of human life. A positive reception of the above aspects is seen in shifting of government and people insight of marine microbes by presenting that the sustainable exploitation of microbial diversity has constructive monetary worth.

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6 Diversity of Microbes in Hot Springs and Their Sustainable Use

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Abstract

Hot spring represents diversified microbial community due to its dynamic natural environment and varied geochemical parameters. Hot spring microbiome produced many novel thermostable enzymes which have enormous industrial as well as biotechnological applications. Moreover, studies on hot spring microbiomes could provide better knowledge about the origin and evolution of earliest life, as they are considered to be most similar to the microorganisms inhabiting the primitive Earth. They have been extensively studied throughout the world mainly by 16S rRNA-based clone libraries along with culture-based methods for discovery of novel thermozymes. These thermostable enzymes have several advantages as they are stable at high temperatures (above 60 °C), tolerate wide range of pH and have less chances of getting contaminated by common mesophilic microorganisms. In addition, thermostable enzymes have other advantages like solvent tolerance, substrate selectivity and stability. In this chapter, an attempt has been made to describe the major industrially important thermozymes like lipase, esterase, protease, cellulase, amylase and xylanase, mostly reported from bacteria and archaea through culturable as well as non-culturable approaches. Many thermophilic bacteria have also been shown to produce biosurfactants which can be a better and cheaper alternative to chemically synthesized ones and are being used in enhanced oil recovery, for controlling oil spills, and can also be used as sources of antibiotics against various food-borne pathogens. Majority of the microorganisms that thrive in hot springs are non-culturable. In this regard, metagenomics has enabled the discovery of novel thermozymes which find applications in biotechnological and pharmaceutical industries.

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Hot springs · Thermophiles · Thermozymes · Biosurfactant · Biofuels

6.1 Introduction

Hot spring microbiome contains several thermotolerant cellular components and thermostable hydrolytic enzymes which have a variety of biotechnological applications. The thermostable enzymes produced by thermophilic microbes include amylases, cellulase, xylanase, protease and several others, which are of extracellular origin and optimally active at relatively elevated temperatures. Thermostable enzymes find applications in various industries (food, detergents, pharmaceuticals, biofuels, etc.). Moreover, microorganisms inhabiting the hot springs are known to play a passive role in silicification. Microorganisms influence the fabric of siliceous sinters that form around the hot spring vents. Thermophiles have different growth patterns which affect the style of laminations, the primary porosity of the sinter and the distribution of diagenetic cementation (Konhauser et al. 2004). Furthermore, the microbial communities of the thermal springs also contain enzymes that are involved in hydrocarbon degradation pathways, which can be employed as promising bioremediation agents in oil spills. Thermophiles metabolize a wide array of organic and inorganic carbon sources, which make them suitable candidates for utilizing a variety of substrates leading to the production of industrially important products. Thermostable enzymes can also be engineered for enhanced activity and to attain higher product yields. The extreme thermophiles have an optimal growth temperature range of 70–100 °C. The genera such as *Caldicellulosiruptor*, *Sulfolobus*, *Thermotoga*, *Thermococcus* and *Pyrococcus* exhibit potentially useful metabolic capabilities like cellulose degradation, metal solubilization and RuBisCO-free carbon fixation (Zeldes et al. 2015). As a whole, the study of hot spring microbiome reveals a vista of extremophilic microorganisms which have immense biotechnological potentials.

6.2 Microbiome in Hot Springs

Hot spring microbial communities are of moderate thermophiles (grow at 50 °C or higher), extreme thermophiles (grow at 65–79 °C) and hyperthermophiles (above 80 °C), which occur in geothermal springs and hydrothermal vents (Wagner and Wiegel 2008). Adaptation in such extreme habitats has evolved the genomic and metabolic flexibility of microbial communities in these ecosystems. As a result, thermophiles have gained importance in the search for novel thermostable proteins or biocatalysts for industrial and biotechnological application (Wang et al. 2014; Fuciños et al. 2014). Microbial diversity in hot springs started with the isolation of thermophilic bacteria by Marsh and Larsen (1953). Subsequently, hot springs at

different geographical location across the globe were extensively studied following 16S rRNA-based clone libraries in combination with cultivation methods (Ward et al. 1998). In general, microbial diversity in hot springs is lower than that in the other environments (Inskeep et al. 2013). Moreover, microbes characterized from such habitats exhibit remarkable genomic plasticity and metabolic diversity (Wemheuer et al. 2013). Thus, exploration of microbial diversity in geothermal environments could provide information on the origin and evolution of earliest life and their potential applications in the translational research (Lewin et al. 2013; López-López et al. 2013).

Among many extreme environments, thermal springs are of considerable interest to researchers worldwide, because they represent unique geothermal environments with respect to their biogeography and physicochemical characteristics that support the growth of a wide range of thermotolerant and thermophilic organisms (Pace, 1997). Thermophiles can grow in the temperature range of 50–121 °C, and they might be the closest living relatives of microbes present in the primitive Earth (Woese et al. 1990). Identification including the determination of phylogenetic position of the culturable bacteria is commonly performed following the methods described in Fig. 6.1. The hot springs, which are mainly concentrated on habitats at low elevations of Earth like Yellowstone National Park (YNP) and Kamchatka Peninsula in Russia and Iceland (Reigstad et al. 2010), Indonesia (Aditiawati et al. 2009) and Malaysia, are the major hotspots for studying thermophilic microbial communities. Among those at higher altitudes, the Himalayan Geothermal Belt (HGB) is rich in hot springs and diversified thermophiles. YNP is the richest habitat of a wide variety of extremophiles, because it contains more than 300 geysers with diverse geochemical properties, temperature and pH (Inskeep et al. 2013). The three main subgroups of the bacterial division *Aquificales* (*Hydrogenobacter*, *Hydrogenobaculum* and *Hydrogenothermus*) are most abundantly found in the thermal springs of YNP along with other bacterial divisions (*Thermotogales*, *Deinococcus* and *Thermodesulfobacteria*). Some of these microorganisms use molecular hydrogen as their primary electron donor, and the others can oxidize sulphide as an electron donor. Archaebacteria accounted for about 50% of the total microflora present in YNP hot springs. An important determinant for microbial diversity in hot springs is the water pH (Hou et al. 2013). *Thermotogae* and *Gammaproteobacteria* are the dominant organisms found in the acidic hot springs near the Mutnovsky volcano located in the southern part of the Kamchatka Peninsula of Russia. *Thermodesulfobacteria*, *Gammaproteobacteria* and *Betaproteobacteria* are the major bacterial genera found in the Uzon Caldera hot spring located in the eastern part of the Kamchatka Peninsula of Russia. The bacterial genera *Euryarchaeota* are predominantly found only in acidic hot springs (Wemheuer et al. 2013). On the other hand, the dominant bacterial genera found in alkaline hot springs are *Thermus*, *Hydrogenobacter* (Hou et al. 2013), *Caldicellulosiruptor*, *Dictyoglomus*, *Fervidobacterium* and *Synechococcus* (Sahm et al. 2013). Among Archaea, Desulfurococcales and Thermoproteales are abundant in alkaline hot springs (Sahm et al. 2013). The microbial diversity of the Jordanian and Malaysian hot springs encompasses many thermophilic bacteria belonging to the

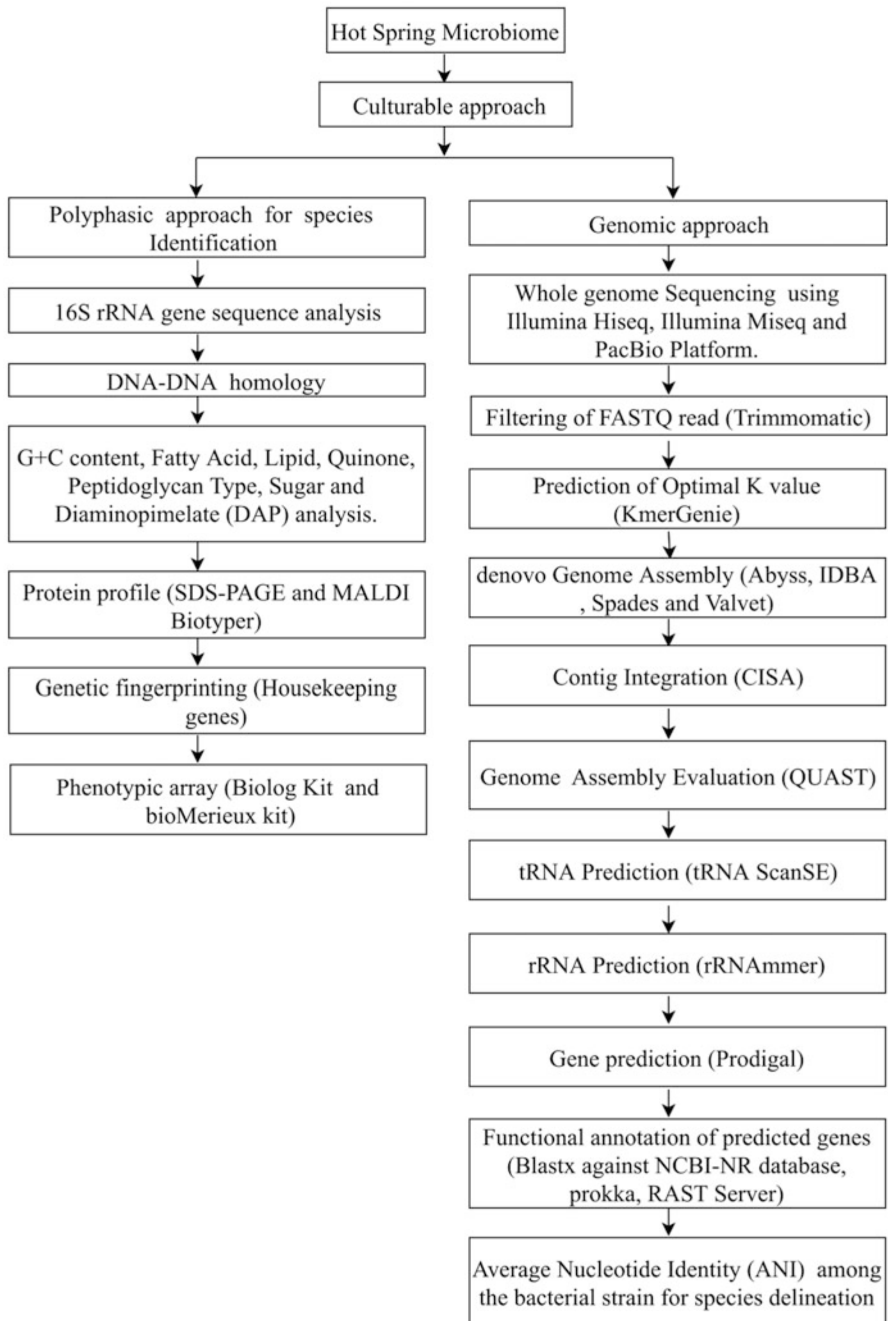

Fig. 6.1 Flow chart proposed for the characterization of culturable bacteria

genera *Bacillus*, *Geobacillus* and *Anoxybacillus* (Al-Batayneh et al. 2011). Furthermore, the microbiome of the HGB falls under three major bacterial phyla, *Firmicutes*, *Proteobacteria* and *Actinobacteria*. Fourteen bacterial genera dominate the HGB microbial population, which are *Anoxybacillus*, *Bacillus*, *Brevibacillus*, *Brevundimonas*, *Burkholderia*, *Geobacillus*, *Paenibacillus*, *Planococcus*, *Pseudomonas*, *Rhodanobacter*, *Thermoactinomyces*, *Thermobacillus*, *Thermonema* and *Thiobacillus* (Sahay et al. 2017). *Thermotoga elfii* (Ravot et al. 1995), *Thermotoga hypogea* (Fardeau et al. 1997), *Thermoanaerobacter uzonensis* (Wagner et al. 2008) and *Herbinix luporum* (Koeck et al. 2016) are the novel thermophilic microbes recorded in the thermal springs across the world. The tropical hot springs are also rich in thermophilic microorganisms among which several new bacterial species such as *Comamonas thiooxydans* and *Gulbenkiania indica* have been identified (Narayan et al. 2010; Jyoti et al. 2010). Hence, it can be concluded that, due to its dynamic natural environment and varied geochemical parameters, hot springs have a potentially diversified microbial community structure. The criteria used in characterizing culturable hot spring bacteria and archaea are listed in Fig. 6.1.

With the advent of cultivation-independent methods, it has been possible to explore the microbial diversity from every ecological niche. In the last two decades, metagenomics has witnessed a powerful tool to access the physiological and biosynthetic pathways of non-culturable bacteria (Wilson and Piel 2013). Following metagenomics approach, several novel enzymes have been identified from hot springs throughout the globe. Thermophilic enzymes primarily were screened from hot springs following culture-based approach, but metagenomics has facilitated the search of new biocatalysts from hot spring microbiome by functional screening of target enzymes in metagenomic libraries. For example, lipolytic enzymes such as lipase and esterase have been characterized by functional screening of the metagenomic library prepared with the DNA samples from Jae Sawn hot spring, Thailand. Both the enzymes were expressed heterologously in *E. coli* which exhibited optimal activity and stability at 70 °C for 120 min (Tirawongsaroj et al. 2008). Kim et al. (2006) using functional screening of the metagenomic library prepared from various environmental soils identified novel esterase of Est25 that has only moderate identity (48%) to known esterases/lipases in the databases. Several novel cellulolytic enzymes with unique activities and/or sequences have been isolated, purified and characterized from metagenomic libraries and showed stability at a wide range of temperatures, pH values and salinity (Voget et al. 2006). Verma and Satyanarayana (2013) retrieved a thermostable xylanase from the metagenome constructed from the DNA extracted from compost soil collected from the vicinity of a hot water spring. Similarly, pectinolytic enzymes such as pectinase that degrade peptic polymers play an important role in plant diseases (Abbott and Boraston 2008). This enzyme produced by phytopathogenic fungi and bacteria is actively involved in the penetration of pathogens and subsequent disease development. Pectinase characterized from metagenome derived from environmental soil samples collected from hot springs of Manikaran (32.0333 N, 77.3500 E), India, and expressed in *E. coli* exhibits more thermostability and can be used in several industries due to its activity over a broad pH range and at elevated temperatures (Singh et al. 2012). The

functional screening of two environmental DNA metagenome libraries obtained from surface of dunes, from the Death Valley Desert (GPS coordinates 36°45′ N, 117°15′ W) and from the Gobi Desert (GPS coordinates 44°18′ N, 110°06′ E), identified two serine proteases belonging to subtilisin (S8A) family and displayed unique biochemical properties. Protease DV1 had an optimum pH of 8.0 and an optimal activity at 55 °C, while protease M30 had optimal activity at pH > 11 and 40 °C, which is potentially useful for biotechnological applications (Neveu et al. 2011). Thus, metagenomic approaches again demonstrate that it is a useful method to retrieve biotechnologically potential biocatalysts from pristine and unique environmental niches. Characterization of non-culturable microbial communities for the screening of biotechnologically important enzymes is shown in Fig. 6.2.

6.3 Biotechnologically Important Enzymes

Many thermostable enzymes with potential biotechnological applications have been reported from Bacteria and Archaea collected from hot springs by several investigators. Several investigations on culturable and metagenomic approaches have revealed not only the presence of genes encoding proteins of biotechnological interest such as amylase, protease, cellulase, xylanase, lipase and esterase, but also to understand the cellular adaptation under extreme conditions. Most of the biocatalysts characterized from extremophiles are superior to their mesophilic counterparts because of their thermal adaptation. Besides, they often provide other benefits such as solvent tolerance, substrate selectivity and stability (Elleuche et al. 2015). Since the cultivation of thermophiles in the laboratory is possible, the methods of recombinant DNA technology will permit cloning genes of interest in mesophilic hosts and their production. Further, temperature and pH play a major role on the stability and catalytic activity of the thermozymes. Effects of pH and temperature on the activity of thermozymes reported from several bacteria are presented in Table 6.1. Thermophiles can be used either in single culture or in consortia for several biotechnological applications such as the production of thermozymes, biosurfactants and biofuels (Urbieta et al. 2015).

6.3.1 Lipolytic Enzymes

Unfortunately, information on the relatedness of the bacterial lipases and esterases studied so far is incomplete and random in the literature. Several studies have been conducted by the elucidation of gene sequences and understanding the resolution of their crystal structures (Cygler and Schrag 1997). In addition, efforts have been made for classification and identification of the lipolytic enzymes into families and subfamilies (Anthonsen et al. 1995, Arpigny and Jaeger 1999). Bacteria produce different classes of lipolytic enzymes that catalyse the cleavage and formation of ester bonds. They are classified into two major families: the carboxylesterases (EC 3.1.1.1) and lipases (EC3.1.1.3) (Arpigny and Jaeger 1999). In general, esterase

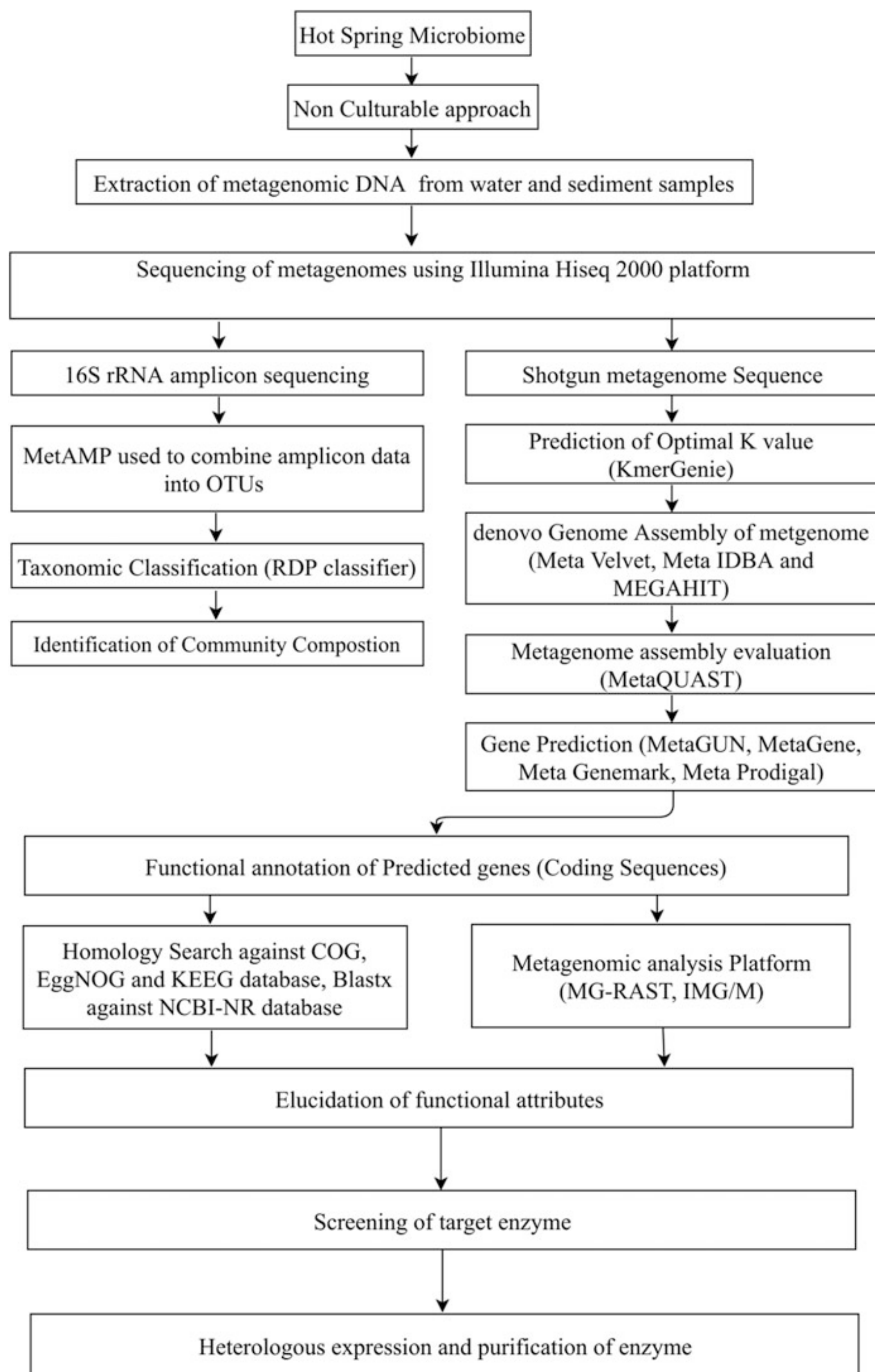

Fig. 6.2 Flow chart proposed for the characterization of non-culturable microbial communities for the screening of biotechnologically important enzymes

Table 6.1 Optimal pH and temperature for the activity of various enzymes

Enzyme	Source	Optimum pH	Optimum Temp. (°C)	References
Lipase	*Thermosyntropha lipolytica* DSM 11003	9.4–9.6	96	Salameh and Wiegel (2007)
	Bacillus sp. L2	9	70	Shariff et al. (2011)
	Bacillus thermoleovorans ID-1	7.5	70–75	Lee et al. (1999)
	Bacillus sp. LBN 2	10	60	Bora and Bora (2012)
	Anoxybacillus sp. HBB16	9.5	55	Bakir and Metin (2017)
Protease	*Bacillus subtilis* BP-36	9	60	Mazar et al. (2012)
	Brevibacterium linens	7.5	50	El-Gayar et al. (2017)
	Bacillus sp. HUTBS71	7.8	65	Akel et al.(2009)
	Bacillus licheniformis LHSB-05	9	50	Olajuyigbe and Kolawale (2011)
	Anoxybacillus mongoliensis	8	60	Namsaraev et al. (2010)
Esterase	*Thermus thermophilus* HB27	≥9	80	Fuciños et al. (2005)
	Anoxybacillus gonensis G2	7.5–9.5	60	Colak et al. (2005)
	Bacillus sp. 4	6	65	Ateslier and Metin (2005)
	Acidicaldus USBA-GBX-499	9	55	Lopez et al. (2014)
Xylanase	*Bacillus sp. strain SPS-0*	6	75	Bataillon et al. (2000)
	Pyrodictium abyssi	6	105	Andrade et al. (2001)
	Bacillus subtilis	6	50	Paula et al. (2002)
	Anoxybacillus flavithermus TWXYL3	7	65	Ellis and Magnuson (2012)
Cellulase	*Anaerocellum thermophilum*	5.0–6.0	85–95	Zverlov et al. (1998)
	Rhodothermus marinus	6.5–8	95	Hreggvidsson et al. (1996)
	Bacillus subtilis	5.0–6.5	65–70	Mawadza et al. (2000)
Amylase	*Thermococcus profundus*	5.5–6.0	80	Chung et al. (1995)
	Desulfurococcus mucosus	5.5	100	Canganella et al. (1994)
	Staphylothermus marinus	5	100	Canganella et al. (1994)
	Thermococcus strain ST489	5–7	80–95	Gantelet et al. (1998)
	Pyrococcus furiosus	5	100	Koch et al. (1990)

is a hydrolase enzyme that splits esters into an acid and an alcohol in a chemical reaction with water (hydrolysis). Primary structures have revealed three families of sequence-related carboxylesterases, namely, the lipoprotein lipase family (L-family), the hormone-sensitive lipase family (H-family) and the cholinesterase family (C-family). Moreover, comparing the structural features of sequences, it appears that lipases, unlike esterases, exhibit a notable difference in the distribution of hydrophobic amino acid residues at the vicinity of their active sites. A wide range of different esterases exists that differs in their substrate specificity, protein structure and biological function (Chahiniana and Sarda 2009). Bacterial esterases and lipases have been classified into eight families based on a comparison of their amino acid sequences and some fundamental biological properties. Enzymes in family I are called true lipases and are further classified into six subfamilies. Enzymes belonging to families II–VIII are esterases (Arpigny and Jaeger 1999).

6.3.1.1 Esterase

Esterases from thermophiles have stability at high temperature in organic solvents and have high potential of utilization in industrial process. In the last two decades, a number of esterases have been characterized from thermophilic archaea and bacteria (Vieille and Zeikus 2001; Zhu et al. 2013). Attempts have been made to characterize thermostable esterases and to identify sequence motifs conserved in the enzyme originated from a broad range of organisms by several authors. Esterase from anaerobic hyperthermophilic archaeon *Aeropyrum pernix K*1expressed in *E. coli* BL21 exhibited optimal catalytic activity at 90 °C. The recombinant protein showed thermostability with a $T_{1/2}$ of 1 h at 100 °C and displayed the activities of both phospholipase A2 and esterase (Wang et al. 2004). Organic solvents are generally used to increase solubility of substrate because it was reported that esterase activity usually remains in organic solvents (Bornscheuer 2002). In this regard, thermostable esterase reported from *Archaeoglobus fulgidus* DSM 4304 showed high thermostability in comparison with other esterases but low enantioselectivity with ketoprofen ethyl ester (Kim et al. 2008). Carboxylesterases are widely distributed in nature and are common in mammalian liver. It has been reported from many thermophiles such as *Sulfolobus acidocaldarius*, *Bacillus stearothermophilus, Sulfolobus shibatae, Sulfolobus solfataricus, Thermotoga maritima, Bacillus acidocaldarius, Thermoanaerobacter tengcongensis*, *Geobacillus thermodenitrificans*, etc. (Charbonneau et al. 2010; Zhu et al. 2013). This enzyme can synthesize acetyl esters when tested in vinyl acetate and toluene (Manco et al. 1998). Further, high mesophilic specific activity of carboxylesterases has been characterized in thermophilic strain *Bacillus stearothermophilus* Tok19A1 (Wood et al. 1995). Esterase from *Sulfolobus tokodaii* strain 7 exhibited high thermostability and showed activity in water-miscible organic solvents such as acetonitrile and dimethyl sulphoxide (Suzuki et al. 2004). Moreover, heterologous expression of esterase gene from hyperthermophilic archaeon *Pyrococcus furiosus* in *Escherichia coli* showed the highest optimum temperature (100 °C) and thermostability ($T_{1/2}$ of 50 min at 126 °C) among esterases reported so far (Ikeda and Clark 1998).

6.3.1.2 Lipase

Lipases (E.C.3.1.1.3) refer to the group of enzymes that catalyse the hydrolysis of triacylglycerol to glycerol and fatty acids. Lipolytic reactions show maximum activity in oil-water interfaces where lipolytic substrates form equilibrium between monomeric, micellar and emulsified states (Verger 1997). On the basis of available data at various protein databases, 47 different lipases were identified which are grouped into six families based on their amino acid sequence homology and 3D crystal structure (Arpigny and Jaeger 1999). Based on their regiospecificity, they are grouped into (i) non-specific lipases, they catalyse triglyceride into free fatty acids and glycerol with mono- and di-glycerides as an intermediate and can remove fatty acids from any position of the substrate; (ii) 1,3-specific lipases, they release fatty acids from 1 and 3 positions of the triglycerides and cannot hydrolyse ester bonds at the secondary positions; and (iii) fatty acid-specific lipases, they show fatty acid selectivity and hydrolyse the esters which have long-chain fatty acids with double bonds at cis position between C-9 and C-10 (Kapoor and Gupta 2012). Lipases contain conserved α/β hydrolase folding pattern. The active site of the enzyme consists of three catalytic residues: a nucleophilic residue (serine, cysteine and aspartate), a catalytic acid residue (aspartate or glutamate) and a histidine residue. Lipase production had been reported from bacteria, fungi, actinomycetes and plants but lipases from microbial origin mainly from bacteria and fungi are widely used in industrial and biotechnological processes. Thermostable lipases have been isolated from various extreme environments such as microbes living in hot springs, industrial effluents and hydrothermal vents. Some of the reported isolates of thermostable lipases include *Bacillus* sp. RSJ-1(Sharma et al. 2002), *Bacillus thermoleovorans* ID-1 (Lee et al. 1999), *Burkholderia ambifaria* YCJ01 (Yao et al. 2013) and *Penicillium simplicissimum* (Gutarra et al. 2009). Thermostable lipases of archaeal origin are very few, viz. *Pyrococcus horikoshii*, which was involved in crude oil refining and was found at 95 °C (Yan et al. 2000). Lipases may exist at 100 °C, but their half-lives are short (Rathi et al. 2000). Thermostable lipases are required in many biocatalytic reactions and have several advantages of carrying out hydrolytic reactions at higher temperature such as high rate of product formation, high dissolution of hydrophobic substrates, increased kinetic energy of reactants and high conversion efficiency (Salihu and Alam 2014). However, lipase activity and specificity tend to be temperature dependent.

Lipases are widely used in food industries to modify flavour by synthesis of esters of short chain fatty acids and alcohols and in leather industries for degradation of emulsified fat on the leather products, in dairy industries for hydrolysis of the milk fat, cosmetics, detergents, pharmaceuticals and in oleo chemical industry. Some of the commercially available lipases are lipolase (*Thermomyces lanuginosus*), Lumafast (*Pseudomonas mendocina*) and Lipomax (*Pseudomonas alcaligenes*). Most of the industrial processes where lipases are used require temperatures above 45 °C such as physical refining of seed oils (Sharma et al. 2002). They have several biotechnological applications such as synthesis of biopolymers like polyphenol and polyesters (Gross et al. 2001), production of biodiesel by using oil from various plant

sources, synthesis of therapeutics and production of agrochemicals (e.g. Indanofan), which is a herbicide used against grass weeds in paddy field; these are mainly done by enzyme immobilization techniques in bioreactors.

Genetic and protein engineering has revolutionized the commercial production of enzymes with improved stability at high temperature, extreme pH, organic solvents and oxidizing agents. Thermostable lipase-producing genes from thermophiles are being cloned and expressed in suitable and fast growing mesophilic host for the biotransformation process (Blackebrough and Birch 1981); *Thermomyces lanuginosus* lipase encoding gene was cloned in *E. coli* DH5α and subsequently expressed in *Pichia pastoris* (Zheng et al. 2011). Today sources of novel enzymes are technically limited as compared with the total microbial diversity pool; the culturable bacteria represent only 1% of the actual microbial diversity in most of the extremophilic environments (Amann et al. 1995). Using functional metagenomic approaches, it is possible to study and identify new lipases from non-culturable microbes from extreme environments like the one isolated from hot springs of Manikaran, India, which has resemblance to lipase from *Geobacillus* sp., which has a high thermostability (Sharma et al. 2012).

6.3.2 Protease

Proteases are a group of enzymes that are able to hydrolyse peptide bonds of long chains of proteins into shorter peptide fragments and eventually into amino acids. They belong to hydrolase class of enzymes (EC 3) and are grouped into the subclass of the peptide hydrolases or peptidases (EC 3.4). Proteases are broadly classified on the basis of three major criteria: (i) type of reaction being catalysed, (ii) nature of the catalytic site and (iii) evolutionarily structure relationship (Barett 1994). They are found in a wide variety of organisms such as plants, animals and microorganisms (bacteria and fungi). Thermostable proteases from microbes have been isolated from various environmental sources such as volcanic areas, hot springs, hydrothermal vents and geothermally heated oil reserves and have been classified into moderate, extreme thermophiles and hyperthermophiles, growing optimally at 50–60 °C, 60–80 °C and 80–113 °C, respectively (Fujiwara 2002, Lasa and Berenguer 1993). They have several advantages such as high stability, increase in rate of reaction and inhibition of microbial contamination, and moreover at higher temperatures unfolded proteinaceous substrate is more susceptible for the thermally stable protease. Since the mid-1980s, a large number of thermostable proteases have been discovered from Eubacteria and hyperthermophilic Archaea such as thermolysin, thermitase and thermomycolin from *Bacillus thermoproteolyticus*, *Malbranchea pulchella* and *Thermoactinomyces vulgaris*, respectively. Some of the known thermostable proteases are as follows: (1) Serine proteases—it comprises the majority of thermostable proteases containing serine residues at the active sites and their inhibitors are di-isopropylflurophosphate (DFP) and phenylmethylsulphonyl fluoride (PMSF). There are two groups of serine proteases,

Group I are like chymotrypsin, while Group II are like subtilisin (Beynon and Bond 1989). They are active at neutral to alkaline pH of 7–11 with molecular masses ranging from 18 to 35 kDa and have broad substrate specificity with esterolytic and amidase activity (Govind et al. 1981). (2) Aspartic proteases—these are acidic proteases and have aspartic acid residues at the active site. They are subgrouped into enzyme-like pepsin and rennin. They show maximal activity at a low pH range of 3–4 and molecular masses ranging between 30 and 45 kDa. (3) Cysteine proteases—their activity depends upon catalytic dyad consisting of cysteine and histidine and are divided into four groups on the basis of their side chain specificity such as trypsin-like with preference for cleavage at the arginine residue, specific to glutamic acid and others. They are active at neutral pH and are susceptible to sulphhydryl agents. (4) Metalloproteases—they are the most diverse of all the proteases and require a divalent metal ion for their activity. They are divided into four groups based on specificity of their action, such as (i) neutral, (ii) alkaline, (iii) myxobacter protease I and (iv) myxobacter protease II and are inhibited by ethylene diamine tetra acetic acid (EDTA).

Proteases from microbes are widely used for removing protein-based stains in laundry detergents, for making cheese, brewing, in food industry and for leather softening. However, only few thermostable proteases are commercially available like Alcalase isolated from *Bacillus licheniformis*. It is useful for recovery of proteins from by-products of meat and fish industry and used for making protein-fortified soft drinks and dietetic food (Synowiecki 2008). Thermostable proteases above 60 °C can be used in dishwashing detergents and for cleaning ultrafiltration membranes (Banerjee et al. 1999; Niehaus et al. 1999). Such proteases can be used to enhance nutritional quality and functionality of protein hydrolysates and minimize their bitterness. Thermolysin is involved in the synthesis of aspartyl-phenylalanine-1-methyl ester commercially known as aspartame and is used as artificial non-saccharide sweetener in food and beverages (De Martin et al. 2001). Proteases are extensively used for research purposes such as the production of Klenow fragments, in cell culture experiments, generation of recombinant antibody, tissue dissociation, exploration of the structure-function relationships by structural studies, removal of affinity tags from fusion proteins in recombinant protein techniques and peptide sequencing. Membrane-bound proteases from *Geobacillus colagenovarans* are useful in improving tissue drug delivery (Nishi et al. 1998). Thermostable proteases from *Thermus* sp. are used for cleaning up of DNA before polymerase chain reaction (PCR) (Bruins et al. 2001).

In the present times, most of the industrially important enzymes are produced by the genetically engineered microorganisms. Thermozymes like proteases have been purified from mesophilic hosts through gene cloning and overexpression. Advantage of using thermostable enzymes is their decreased activity at low temperatures, and this feature can enable us to terminate the reaction just by cooling, and thus, it avoids the use of chemical inhibitors and expensive enzyme-removing procedure at the end. Several reports are available on the isolation and genetic manipulation of microbial proteases such as (i) enzyme overproduction, (ii) study the structure of the protein

and its role in pathogenicity and to develop therapeutics against them and (iii) identify the active sites of the enzyme through protein engineering and alter the enzyme properties for commercial applications.

Thermostability of enzyme is important in detergent and leather-processing industries. In this regard, enhancement of thermostability of the enzyme is possible by protein engineering following the replacement of specific amino acid residues by site-directed mutagenesis. For example, subtilisin E, a non-specific protease obtained from *Bacillus subtilis*, does not have cysteine residue; however, by introducing the cysteine residue, it appeared to form a disulphide bond and showed more thermal stability and catalytic activity to that of wild-type enzyme (Takagi et al. 1990). With the development of metagenomics, it has been possible to explore the new enzymes produced by uncultivable microorganisms and their biotechnological applications. For example, thermotolerant alkaline protease CHpro1identified from hot spring metagenome collected from Ladakh shows high range of alkaline stability that can be used in detergent industries (Singh et al. 2015).

6.3.3 Polysaccharide-Degrading Enzymes

Polysaccharides are carbohydrates made of monosaccharides and on hydrolysis produce monosaccharides or oligosaccharides. The structure of polysaccharides varies from linear to highly branched. Due to the flexible structure of the carbohydrate polymer, the biocatalysts have been evolved through breakdown of glycosidic backbones (Varki et al. 1999). Examples of polysaccharides are starch, glycogen, cellulose, chitin, etc. These are amorphous or insoluble in water.

6.3.3.1 Cellulase

Cellulase refers to a group of enzymes that hydrolyse β-1, 4-glycoside bond present inside the cellulose polymers by acid catalysis mechanism. Cellulases break the cellulose molecule into polysaccharides, oligosaccharides and monosaccharides such as β-glucose. Cellulase is divided into catalytic domain and regulatory domain. The catalytic domain is further classified into numerous families based on their amino acid sequences and crystal structures (Henrissat and Bairoch 1993; Parkkinen et al. 2008; Vlasenko et al. 2010). Organisms like bacteria, fungi and algae have been reported from extreme environments and are known to produce cellulase for commercial use (Ja'afaru 2013). Further, cellulases have been categorized under different types (i) Thermostable cellulase**:** Thermostable enzymes are mostly produced by thermophilic and mesophilic organisms. Generally, thermophilic microorganisms such as *Acidothermus cellulolyticus, Rhodothermus marinus* and *Anaerocellum thermophilum* (Zverlov et al. 1998) are potential sources for thermostable enzymes. Thermostable enzymes in the hydrolysis of lignocellulosic materials have several advantages such as higher specific activity, higher stability and increased flexibility for the industrial bioprocesses. Thus, carrying out the hydrolysis at higher temperature would ultimately lead to improved performance, i.e. decreased enzyme dosage

and reduced hydrolysis time and, thus, potentially decreased hydrolysis costs. (ii) Acidophilic cellulase: Cellulases which performs optimally under extreme pH (below pH 4.0) are considered as acidophilic cellulase. Most of the acidophilic cellulases have been reported from thermophilic bacteria (Gomes and Steiner 2004). Although structure and adaptive mechanisms of acidophilic cellulases are poorly understood, most of them are associated with thermostability. There are few reports in *Archaea* family producing cellulase with optimum pH of 4; however, in case of bacteria, such types of cellulase are elusive. (iii) Alkaliphilic cellulase: Alkaliphilic cellulases are stable and perform optimally at pH 9. Horikoshi (1999) reported bacterial isolates (*Bacillus sp*. strains N4 and 1139) producing extracellular alkaline cellulase. Despite industrial importance of alkaline cellulase, the structural part is poorly understood. As most of the Alkaliphilic bacterial cellulases are extracellular, structural modification adopted by these enzymes to perform under extreme pH needs to be investigated (Fukumori et al. 1985; Madren et al. 2000).

6.3.3.2 Amylases

Amylases are capable of hydrolysing starch into various α-limit dextrin-containing α (1–6) glycosidic bonds and a mixture of malto-oligosaccharides, glucose and maltose (Yang and Liu 2004). Further, amylase has been classified based on the hydrolysis of the glycosidic bonds, such as endoamylase (α-amylase), exoamylase (β-amylase, glucoamylase) and debranching enzymes (pullulanase, isoamylase). It occupies about 25–33% in the world enzyme market (Ashwini et al. 2011). Amylases play a significant role in nutritional, cosmetic and pharmaceutical processes (Nigam and Singh 1995), and with the recent developments in biotechnology, its usage has expanded to many sectors such as medicinal, clinical and analytical chemistry, in starch saccharification and in the textile, food, brewing and distilling industries (Gupta et al. 2003; Kandra 2003). Various sources of amylases are plants, animals and microorganisms. An advantage of choosing microorganisms (fungi, yeasts and bacteria) over plants and animals is to have bulk production capacity which is economically beneficial and easy to manipulate to obtain an enzyme of preferred requirement.

α-Amylase (E.C.3.2.1.1) is one of the important enzymes having wide applicability in various sectors such as food, fermentation, textile, paper, detergent and pharmaceutical industries. The initial hydrolysis of starch is mainly catalysed by α-amylase into short oligosaccharide of endo-amylase family, by cleaving α-D-(1–4) glycosidic bond (Iulek et al. 2000, Kandra 2003). For complete hydrolysis of starch and other polymers, it requires the activity of other amylases such as glucoamylase or pullulanase. It has a three-dimensional structure, containing highly specific catalytic group, which promotes the cleavage of α-D-(1–4) glycosidic bonds (Iulek et al. 2000). Starch is one such example which is an essential part of human diet and therefore being processed into a variety of products such as starch hydrolysate, glucose syrups, etc., used in food industry. Sugar obtained after the hydrolysis of starch is highly dependent on the enzyme origin, temperature and hydrolysis condition. In short, specificity, thermostability and pH response of enzyme have a critical role in industrial use (Kandra 2003).

The two methods to produce α-amylase are (i) submerged fermentation method (SmF) and (ii) solid-state fermentation (SSF), both of which are dependent on a variety of physicochemical factors. SmF is a traditional method of producing industrially important enzyme, which makes it easy to control the different parameters such as temperature, pH, oxygen transfer and moisture and aeration (Couto and Sanromán 2006; Gangadharan et al. 2008), whereas SSF provides natural environment for microorganism to grow and therefore is a preferred choice to produce useful value-added products. Theoretically, yeast and fungi are considered as suitable microorganisms, whereas bacteria are considered unsuitable due to its high water activity. But previous reports suggest that the bacteria can be manipulated and managed for SSF process (Pandey 2003). Recently, researchers have found that the SSF is a suitable method for the production of enzymes and other thermolabile products especially when higher yield is desired in comparison with SmF (Couto and Sanromán 2006; Tanyildizi et al. 2007). Besides fermentation conditions, the properties of each enzyme such as thermostability, pH and calcium independency must match the application. Such as in the starch industry, α-amylase used should be active at low pH, whereas in detergent industry, it should be active at high pH. Other notable factors are growth medium composition, pH, concentration of phosphate, age of inoculum, temperature, aeration, carbon and nitrogen source (Couto and Sanromán 2006).

Majority of enzymes being used were obtained from mesophilic organism thus limiting their use as they were unstable at high temperature and pH and ionic strength (Hough and Danson 1999). Therefore, thermophiles came into picture as it is a preferable source of thermostable enzymes having optimum activity at extreme temperature (50 °C or above) (Brown and Kelly 1993), thus being used for commercial purpose. Thermophiles can be isolated from a number of marine and terrestrial geothermally heated habitats including shallow terrestrial hot springs, hydrothermal vents, sediment from volcanic islands and deep sea hydrothermal vents. The potential benefits of these natural thermophiles are not only in production of glucose syrup but also for the synthesis of non-fermentable carbohydrates, anti-cariogenic and anti-staling agents for baking (Bauer et al. 1998). Other benefits of carrying out the biotechnological processes at comparatively higher temperature are that it reduces the contamination risk by common mesophilic microorganisms. Significant impact of working at elevated temperature can be seen in the bioavailability and solubility of organic compounds, thereby providing efficient bioremediation (Becker et al. 1997).

Hyperthermophilic microbes generally produce enzymes in low titres. To overcome this, a number of genes encoding amylolytic enzymes from thermophiles are cloned and expressed in mesophiles (Bertoldo and Antranikian 2001). The thermostable protein expressed in mesophiles retains thermostability, folds correctly at low temperature, resistant to host proteolysis and purified easily by thermal denaturation process, thus maintaining the degree of enzyme purity, which is generally required in industrial applications.

Thermo-active amylolytic enzymes have been reported in hyperthermophilic Archaea of the genera *Sulfolobus*, *Thermophilum*, *Desulfurococcus* and *Staphylothermus* (Bragger et al. 1989). The most thermo-active α-amylases have been

characterized from hyperthermophilic Archaea *Pyrococcus furiosus* and *Thermococcus hydrothermalis* (Lee et al. 1996; Dong et al. 1997). They have optimum activity at temperature between 80 and 100 °C. Amylases such as glucoamylase have been reported from thermo-acidophilic Archaea such as *Thermoplasma acidophilum*, *Picrophilus torridus* and *Picrophilus oshimae*, having optimal activity at pH 2.0 and 90 °C. Likewise, there are numbers of other hyperthermophilic, thermo-acidophilic and thermo-alkaliphilic microorganisms that belong to archaeal and bacterial groups that produce amylolytic enzymes such as α-amylase, α-glucosidase, pullulanase, glucoamylase and others.

6.3.3.3 Xylanase

Xylanase (EC 3.2.1.8) is one among the industrially important enzymes with an increasing demand in market. It is a hydrolytic enzyme (*O*-glycoside hydrolases) which cleaves at β-1, 4-xylosidic linkage of xylan, an important constituent of plant cell wall. Xylan is a heteropolysaccharide containing *O*-acetyl, arabinosyl and 4-*O*-methyl-D-glucuronic acid substituents and accounts for approximately one third of all renewable organic carbon on Earth (Prade 1995). It is a major hemicellulosic constituent, and it makes the major polymeric constituent of plant cell wall together with cellulose and lignin (Kulkarni et al. 1999). It has a role in fibre cohesion and cell integrity (Beg et al. 2001). Because of the complexity of xylan, there are diverse xylanases with varying specificities, primary sequences and folds and hence has led to limitations with the classification of these enzymes by substrate specificity alone. A complete classification system has been introduced (Teplitsky et al. 2000), which allows the complete classification of xylanases based on their primary structure comparisons of the catalytic domains only and groups enzymes in families of related sequences (Henrissat and Coutinho 2001). The number of xylanase family continues to grow as new glycoside sequences are being identified. At present, a total of 14 different clans have been made (GH-A to GH-N), each clan containing approximately 2–3 families, with an exception of clan GH-A containing almost 17 families. Glycoside hydrolase families 5 (GH-A), 7 (GH-B), 8 (GH-M), 10 (GH-A), 11 (GH-C) and 43 (GH-F) have endo-1,4-β xylanase activity because of their truly distinct catalytic domain.

Variety of microorganisms including bacteria, actinomycetes, yeast and fungi produce xylanases. Fungal and bacterial xylanases which have optimal activity at or close to mesophilic temperatures (40–60 °C) and neutral or slightly low pH are considered. Previous reports suggest that xylanase activity and stability are found at wide pH range of 2–11 and temperatures 5–105 °C (Collins et al. 2005) and high salt concentrations of about 30% (Waino and Ingvorsen 2003). Under natural conditions, xylanolytic enzymes seem to be induced by the product of their own action. The best inducer for xylanase production seems to be xylan (Walia et al. 2014). High molecular weight of xylan does not pass through the cell wall; therefore, low molecular fragments of xylan (xylobiose, xylotriose, xylooligosaccharides of xylose) are used to induce xylanases (Walia et al. 2013).

Microorganisms produce a variety of xylanases that have formed a major group of industrial enzymes capable of degrading xylan into renewable fuels and chemicals (Hatanaka 2012) and their application in food, paper and pulp industries (Singh et al. 2013). To bring down the pollution and to enhance the quality of pulp, green biotechnology is being approached in recent years. Use of cellulase-free xylanase enzyme in pre-bleaching is environmentally benign and economically cheap technology, which decreases the required quantity of chemicals used for bleaching in order to achieve brightness in subsequent phases. Enzyme pretreatment enhances the dissemination of sodium hydroxide in both hardwoods and soft woods, as well as conventional pulping of wood chips and pulp uniformity (Woldesenbet et al. 2012). Three thermotolerant bacteria, namely, *Thermobacillus* sp. NBM6, *Paenibacillus ehimensis* NBM24 and *Paenibacillus popilliae* NBM68, isolated from hot springs of Indian Himalayas, were found to produce cellulase-free xylanase (Sahay et al. 2017). Thus, thermostable enzyme isolated from thermophiles is known to have a wide application in both commercial as well as industrial processes because of their inherent stability to withstand extreme conditions of industries (Verma et al. 2015). In recent years, xylanase has pulled attention of many researchers due to its wide application in biotechnology such as pre-bleaching of pulp, improving the digestibility of animal feed stocks, modification of cereal-based stuffs, bioconversion of lignocellulosic materials and agro-wastes to fermentable products, clarification of fruit juices and degumming of plant fibres.

6.4 Thermophiles as a Potential Source of Biofuel Production

Global energy crisis and the limited supply of nonrenewable energy resources have rekindled the worldwide focus towards renewable energy production. With fossil fuels predicted to be on the verge of depletion by 2081, renewable means of energy production, more specifically by biological means, becomes pretty indispensable. Biofuels possess the advantages of being environmentally benign, biodegradable and a renewable source of energy. Lignocellulosic wastes can be used as a potential substrate for biofuel production (second-generation biofuels) because it contains cellulose and hemicellulose, which are abundant sources of fermentable carbohydrates (Bhalla et al. 2013). To access the cellulosic and hemicellulosic fractions, the lignocellulose deconstructing enzymes, cellulases and hemicellulases are required to release the fermentable sugars (Yeoman et al. 2010). The drawbacks associated with the existing enzymatic hydrolysis techniques of lignocellulosic biomass include slow enzymatic hydrolysis rates, requirement of high dosages of enzymes, low yields of sugars from the lignocellulosic biomass, etc. (Viikari et al. 2007). To overcome these gridlocks, thermophilic microorganisms are used for producing biofuels from cellulosic raw materials. Thermophiles are found in various geothermally heated regions of the Earth, such as in hot springs and deep sea hydrothermal vents, as well as in decaying plant

matters, such as peat bogs and compost. The advantages of using thermophiles encompass their ability to survive and thrive at a wide temperature range of about 40 °C to even more than 100 °C. In addition to this, microorganisms growing at temperatures of 50–80 °C are sources of highly active and thermostable enzymes (Zambare et al. 2011; Liang et al. 2011). These enzymes offer several advantages in the hydrolysis of lignocellulosic materials, viz. increased solubility of reactants and products, resulting in higher reaction velocities thereby decreasing the enzyme dosage, reduction of hydrolysis times (Viikari et al. 2007), increased productivity, facilitated recovery of volatile biofuels (e.g. ethanol, butanol, etc.), etc. (Taylor et al. 2009). Thermophilic microbes can efficiently produce thermostable cellulases and xylanases which help in the degradation of the lignocellulosic biomass (Liang et al. 2011).

Catalysis of depolymerization of cellulose is carried out by the cellulase enzyme. Cellulase aids in the efficient utilization of crystalline cellulose by the microbes (Arantes and Saddler 2010). Examples of thermophilic fungal genera which can produce thermostable cellulases are *Sporotrichum thermophile*, *Chaetomium thermophilum*, *Coniochaeta ligniaria*, etc. (Barnard et al. 2010). Furthermore, several thermophilic bacteria belonging to the genera *Bacillus*, *Geobacillus*, *Acidothermus*, *Caldocellum*, etc., produce thermostable cellulases. Hyperthermostable lignocellulolytic enzymes (optima above 80 °C) can be produced from the bacterial genera *Thermotoga* (Bok et al. 1998), *Anaerocellum* and *Rhodothermus*, and archaeal genera *Pyrococcus* and *Sulfolobus* (Huang et al. 2005). Moreover, thermophilic microbes and thermostable enzymes are resistant to a wide range of pH which renders them as a highly appreciable candidate for extremophilic biofuel production. During biofuel production, the acid or alkali pretreatment of the lignocellulosic biomass to remove the lignin sheath is necessary, which can be avoided by the applications of thermo-acidophilic and thermo-alkaliphilic enzymes produced by the thermophiles (Bhalla et al.2013). On the other hand, complete enzymatic hydrolysis of hemicellulose fractions requires the synergistic action of a group of thermostable xylanase enzymes produced at high temperatures by the thermophiles and hyperthermophiles. Thermophilic fungi that can produce thermostable xylanases include *Laetiporus sulphureus* (Lee et al. 2009), *Talaromyces thermophilus*, *Nonomuraea flexuosa*, etc. (Bhalla et al. 2013). The thermophilic bacteria which can efficiently produce thermostable xylanases to degrade the hemicelluloses belong to the genera *Thermoanaerobacterium*, *Thermotoga*, *Alicyclobacillus*, *Anoxybacillus*, *Cellulomonas* and others.

By virtue of these advantages of the thermophiles and hyperthermophiles, they are treated as highly efficient candidates for different types of biofuel production, such as biohydrogen, bioethanol, biobutanol, biomethane, etc. Thermophiles can utilize a wide range of carbon sources for biofuel production, and the risk of contamination in thermophilic bioprocessing is also considerably less because of their high operational temperature. Thermophiles can also be lucrative candidates for metabolic engineering to enhance the product yield because they contain many thermostable genes that can be engineered (Jiang et al. 2017).

6.5 Biosurfactants from Thermophiles

Microbial surfactants (biosurfactants) are amphipathic molecules having molecular weights in the range of 500–1500 Daltons. These are made up of peptides, saccharides or lipids or their combinations. In biosurfactants, the hydrophobic moiety is either a long-chain fatty acid, hydroxyl fatty acid or α-alkyl-β-hydroxy fatty acid, and the hydrophilic moiety may be a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid or an alcohol (Luna et al. 2009). Among biosurfactants, the lower molecular weight glycolipids and glycopeptides are typically more effective in reducing surface and interfacial tensions while high molecular weight amphipathic polysaccharides and proteins act as oil-in-water stabilizers. Microbial surfactants play crucial roles in different physiological functions, which include their positive effects in the solubility of water-insoluble compounds, binding of heavy metals, bacterial pathogenesis, cell adhesion, quorum sensing, production of antimicrobial compounds, etc. (Cortes-Sanchez Ade et al. 2013; Gudina et al. 2010). Biosurfactants also have antibacterial, antifungal and antiviral properties, by virtue of which they can be used as an alternative to conventional antibiotics against various food-borne pathogens (Sharma and Singh 2014; Banat et al. 2010). Biosurfactants have several advantages over the chemically produced surfactants, which encompass their biodegradability, low toxicity, less production cost due to cheaper raw materials and effectiveness at extreme conditions of temperature, pH and salinity (Pyaza et al. 2006). Biosurfactants are used in enhanced oil recovery (EOR) technique, controlling oil spills, detoxification and biodegradation of oil-contaminated industrial effluents and soils (Bryant 1987; Ron and Rosenberg 2002). Biosurfactant production by microbes are dependent on several parameters, which include carbon source, nitrogen source, carbon-to-nitrogen ratio (C:N), temperature, pH, etc. (Fakruddin 2012). Though most of the biosurfactants are produced from bacteria under mesophilic conditions, some thermophilic bacteria can also efficiently produce biosurfactants. A thermotolerant biosurfactant producing *Bacillus* sp. was isolated by Banat (1993) that grows at around 50 °C temperature on a hydrocarbon-containing medium and suitable for use in microbial-enhanced oil recovery (MEOR) technique and oil-sludge clean up (Banat 1993). Biosurfactants from thermophilic *Streptococcus thermophilus* was isolated by Busscher et al. (1996), which can be employed for establishing an anti-adhesive, non-fouling coating for heat-exchanger plates in pasteurizers in the dairy industry (Busscher et al. 1996). In-situ microbial oil recovery was enhanced by the application of the biosurfactants isolated from *Bacillus licheniformis* JF2 which had both thermotolerant and halotolerant characteristics (Jenneman et al. 1983). *Methanobacterium thermoautotrophicum*, *Halobacterium halobium* and *Sulfolobus solfataricus* are examples of thermophilic bacteria which have emulsifying activity. These thermophilic bacteria produce cell-associated emulsifiers which in turn reduce the capillary forces that entrap oil within the pores of the rock, and the sweep efficiency of a water-flood can also be enhanced

by these emulsifiers. Bioemulsifiers can also stimulate oil production in marginal wells that have approached their economic limit of operation (Trebbau de Acevedo and McInerney 1996). Some strains of *Bacillus subtilis* are also able to synthesize biosurfactants under thermophilic conditions (Makkar and Cameotra 1997). Since the temperatures of the oil coops are almost more than 60 °C, thermophilic biosurfactants find its potential applications in the microbial oil recovery processes from oil coops.

6.6 Conclusions and Future Perspectives

Enzymes are known for their efficiency, selectivity and increasing the rate of reaction, thus offer more competitive processes than chemical catalysts. Isolation of novel species from environments with high temperatures has the potential to tolerate extreme temperature stresses of industrial process, thus provide additional advantage over mesophilic organisms. Several studies have been conducted in recent years to look for microbial diversity in environments with extreme high temperatures. Enzymes isolated from the thermophilic microorganisms provide several advantages like lower cost, recovery of high product, provide an ease to genetic manipulation, regular availability and provide rapid growth over plant- and animal-derived product. These extremozymes are produced from a wide range of microbial sources such as bacteria, *Actinobacteria* and fungi. Some of the extremozymes known to have a wide applicability in many biotechnological processes are amylase, lipase, xylanase, cellulose and protease. These biocatalysts are having a wide applicability in biotechnological industries such as paper, pulp, food, textile, beverages, pharmaceutical, cosmetics, feedstuff, for fuel production and chiral substances in the chemical industry.

With the advances in the areas of genetic and protein engineering, it is possible to produce enzymes to suit industrial processes, thus resulting not only resulting in development of number of new products but also improving the process and performance level of already existing ones. As stated earlier, though enzymes are having a great potential, their industrial application was being hampered mainly because of their undesirable properties such as stability, catalytic efficiency and specificity. Attempts are being made to obtain enzymes from natural resources by random mutation, directed evolution and immobilization (Elleuche et al. 2014). The process of random mutation, followed by screening of a library, has led to rapid and extensive enhancement of various properties of enzymes and enantioselectivity in biocatalyst industries. Several industries demand for alternative green technologies for replacing chemical processes with cleaner, safer and ecofriendly biocatalytic processes.

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7 Thermophilic Fungal Diversity in Sustainable Development

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Abstract

Thermophilic fungi inhabit a great variety of ecosystems such as soils, composts and several others. They have also been isolated from non-thermogenic environments. Both morphological and molecular approaches have been employed in identifying them. These fungi degrade a large number of lignocellulosic and other biomasses by producing a wide range of varied hydrolases. Hydrolytic enzymes of thermophilic fungi exhibit unusual properties, for example, thermostability, tolerance to organic solvents, long shelf life and others required for applications in different industrial processes. These moulds play a key role in composting, thus in mushroom production. Various antimicrobials and secondary metabolites are well known to be produced by these organisms, besides being useful in generating nanoparticles. They are also used as single cell protein and in waste treatment and bioremediation. The centre of attention in this chapter is the diversity of thermophilic fungi and their potential utility in sustainable development.

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Thermophilic fungi · Degradation · Lignocellulosics · Enzymes · Composting · Single-cell protein · Bioremediation

7.1 Introduction

Only a few species of fungi among all eukaryotic organisms have the potential to multiply at higher temperatures ranging from 45 to 55 °C, and these fungi include thermotolerant and thermophilic forms randomly differentiated on the basis of their maximum and minimum growth temperatures (Cooney and Emerson 1964). Thermophilic fungi are defined as those having minimum growth temperature at 20 °C or above and maximum at 50 °C or above, whereas thermotolerant fungi have temperatures for growth from below 20 to 55 °C (Blochl et al. 1997, Brock 1995). The majority of eukaryotes fail to grow under prolonged exposure at 40–45 °C (Alexandrov 1977), and out of 1,40,000 recorded fungal species, approximately only some 30 species are capable of breaking the upper limit of temperature of the eukaryotes. Among microflora, thermophilic fungi are the major group that thrives in piles of forestry and agricultural products, heaped mass of plant materials and other organic matter accumulated wherein a humid, aerobic and warm environment supports all fundamental conditions for growth and development (Allen and Emerson 1949; Miehe 1907).

There are definitions for other thermophilic organisms which do not hold well for thermophilic fungi because of their varied temperature tolerance limits. Because of variation in growth temperatures of different organisms, thermophilic fungi must be defined distinctly (Salar 2018). There are some common terms other than thermophilic and thermotolerant, which are used with reference to thermophily in fungi:

- **Transitional thermophiles:** The group of fungi that grow below 20 °C and can also resist a temperature just near 40 °C (Apinis and Pugh 1967).
- **Thermophilous fungi:** Those fungi capable of growing at elevated temperatures and this term involves both thermophilic and thermotolerant fungi with optima at or above 40 °C (Apinis 1963).
- **Thermoduric fungi:** Those fungi that have normal growth temperature (22–25 °C) but their propagules can resist temperatures close to 80 °C (Apinis and Pugh 1967).
- **Poikilophilic or poikilotrophic fungi:** Those fungi that tolerate conditions caused by extreme environmental stresses (Brock and Fred 1982).
- **Poikilothermic fungi**: Those organisms that can tolerate extremes of fluctuating temperatures (Brock and Fred 1982).
- **Eurythermal fungi:** The organisms which can survive and grow at a wider temperature range and generally found in environments with varying temperatures (Brock and Fred 1982).

Thermophilic fungi have been reported in large heterogeneous habitats, natural as well as artificial. In nature, there are several extreme environments like environments with low and high temperatures, high acid, high salt, high alkali, high pressure and radiation and drought conditions. The thermophilic fungi also occur in soils of different kinds and habitats having decomposed residues of plants like hay piles, composts, woodchip piles, stored grains, nesting stuff of animals and birds, municipal waste and heap of other organic material in humid, aerobic and warm environments that provide fundamental physiological conditions for growth and development. These fungi can tolerate a range of pH between 4.0 and 8.0. *Rhizomucor miehei* and *Rhizomucor pusillus* colonize hay with pH 6.5, while *Thermomyces lanuginosus* and *Talaromyces thermophilus* colonize hay with pH near neutral (Rosenberg 1975). Thermophilic moulds are receiving attention in industrial biotechnology because of their growth at elevated temperatures. The major areas in which thermophilic fungi have been studied worldwide are protein engineering and enzyme technology. Now it is well recognized that activities at elevated temperatures are generally related to thermostability of proteins. The thermophilic fungi produce proteins which tend to be more thermostable than those of mesophiles. The advantage of using thermophilic fungi in industrial processes is that they solve the problem of temperature maintenance at optimal levels during the entire period of cultivation. Biotechnological capabilities of the thermophilic fungi have been noticed because of their efficiency to decompose organic matter, act as natural scavengers and bio-deteriorants and produce intra- and extracellular compounds such as enzymes, antibiotics, polysaccharides, amino acids, phenolic compounds sterols, SCP and nutritionally enriched feeds. They are also suitable agents for bioconversions, for example, they have a role in mushroom compost preparation. They are involved in genetic manipulations; this is a recent development. Investigations carried out by Cooney and Emerson (1964), Tansey and Brock (1978), Sharma (1989), Sharma and Johri (1992) and Singh and Satyanarayana (2014, 2016) suggested that thermophilic fungi are nature-borne biotechnologists. In the economy of nature, thermophilic fungi have been playing an important role ever since they evolved on Earth. This chapter discusses investigations on thermophilic fungi such as their occurrence, diversity, physiology, biochemistry and their potential biotechnological applications as they are ubiquitous and have immense potential in industrial biotechnology and environmental management.

7.2 Occurrence and Ecology

On Earth, diversity is a sure sign of life. About 1,40,000 species of fungi have been described by microbiologists so far, and every year, researchers are adding hundreds of new fungal species. Thermophilic fungi are found in diverse habitats such as in high temperature, salt, alkali, acid, pressure and radiation environments and in drought conditions (Fig. 7.1). Although these conditions are unfavourable for survival of most of the organisms, because of natural selection for a long time,

Fig. 7.1 Different natural habitats of thermophilic fungi

some microorganisms have adapted to survive and grow in these conditions. Thermophilic fungi occur in various habitats and soils where decomposing plant materials are found (piles of hay and woodchips, composts and bird and animal nesting materials), which provide fundamental physiological situation for the growth and development of fungi (Singh and Satyanarayana 2009a, b). In tropical countries, soils do not contain higher populations of thermophilic fungi than the soils in temperate countries. In acidic thermal environments, thermophilic fungi are more common than those in alkaline conditions (Tansey and Brock 1972). Habitats of thermophilic fungi reported in literature are listed in Table 7.1.

Soil is considered as a repository of all life forms where most changes occur, which enable life to continue. Soil is also a natural habitat for thermophilic fungi (majority in the upper layer rich in organic matter). Waksman et al. (1939) isolated thermophilic fungi from soil. The other potential habitat of thermophilic fungi is coal spoil tips (Evans 1971a, b). From coal spoil tips, 32 thermotolerant and thermophilic fungi have been isolated, and it was observed that the group of thermophilous fungi was higher in well-colonized warm areas than the group of fungi in corresponding non-warm areas. Similarly, Ward and Cowley (1972) and Taber and Pattit (1975) reported forest soils of loblolly pine, pinescrub oak, floodplain hardwood, pine hardwood and peanut soils as potential habitats of thermophilic fungi. In aquatic sediments, Tubaki et al. (1974) initially studied the existence of thermophilic fungi in rivers and lakes, and it was mysterious in view of the little oxygen level (average

Table 7.1 Habitats of thermophilic fungi (Singh and Satyanarayana 2008)

Sources	References
Thermogenic habitat	
Composts	Miehe (1907), Straatsma et al. (1991), Straatsma and Samson (1993), and Singh (2007)
Soil	Vartaja (1949), Tansey and Brock (1973), Morgan-Jones (1974), Friedman and Galum (1974), Tansey and Jack (1976), (1977), Jaitly and Rai (1982), Hassouni et al. (2006), Singh and Satyanarayana (2006), Salar and Aneja (2006), (2007), and Singh (2007)
Hay	Miehe (1907)
Paddy straw compost	Satyanarayana and Johri (1984) and Singh (2007)
Wheat straw compost	Chang and Hudson (1967), Moubasher et al. (1982), and Singh (2007)
Mushroom compost	Fergus (1964), Fergus and Sinden (1969), Fergus and Amelung (1971), Straatsma et al. (1991), and Wiegant et al. (1992)
Municipal compost	Crisan (1959), (1969), Cooney and Emerson (1964), Eggins and Mills (1971), Barnes et al. (1972), Malik and Eggins (1972), Brown et al. (1974), Mills and Eggins (1974), Champman et al. (1975), Subrahmanyam et al. (1977), Tansey and Jack (1977), Matsuo et al. (1977), Matsuo and Yasui (1985), and Gomes et al. (1993)
Coal spoil tips	Apinis (1963), Evans (1971a, b), (1972), and Johri and Thakre (1975)
Stored grains	Christensen 1957, Clarke et al. (1969), Mulings and Chesters (1970), Awao and Mitsugi (1973), Flanningan (1974), Mehrotra and Basu (1975), Taber and Pattit (1975), and Davis et al. (1975)
Non-thermogenic habitats	
Cooling towers	Subrahmanyam (1978)
Dust of library books	Subrahmanyam (1978)
Arctic and Antarctic soils	Ellis (1980a, b)
Himalayan soil	Sandhu and Singh (1981)
Human skin	Subrahmanyam et al. (1977)
Birds' nests	Cooney and Emerson (1964), and Satyanarayana et al. (1977)
Air	Hughes and Crosier (1973)

10 ppm, <1.0 ppm at a depth of 31 m) and lower temperature (6–7 °C) available at the bottom of lakes. Mahajan et al. (1986) reported that some thermophilic fungi can survive under stress conditions like absence of oxygen, water pressure and desiccation. For fungal development, atmosphere, humidity and temperature of these domains are beneficial (Salar and Aneja 2007). Maheshwari et al. (1985) isolated a thermophilic fungus, *Melanocarpus albomyces,* at 50 °C from soil and compost by supplementing liquid medium with sorbose, glucose and asparagine, followed by enrichment culture in sugarcane bagasse. Gomes et al. (1992) isolated a thermophilic fungus related to *Deuteromycetes* from jute cuttings piled for maturation after water/oil emulsion having optimum temperature at 55 °C and pH at 6.0 and that grew in modified Mandels' medium. Thermophilic and thermotolerant fungal

strains, *Rhizomucor pusillus* (Lindt) Schipper, *Rhizopus microsporus* van Tieghem and *Aspergillus fumigatus* Fresenius were isolated by Cordova et al. (2003) in Mexican tropical and subtropical regions from soil and coconut residues; these fungi were capable of growing at temperatures above 50 °C. *Humicola* sp. SKESMBKU03 was isolated from horse dung manure collected from Hyderabad, Telangana, by growing in YpSs medium (Kumar et al. 2014) at 45 °C. *Thermomyces lanuginosus, Myriococcum thermophilum, Malbranchea cinnamomea, Thermoascus aurantiacus, Scytalidium thermophilum, Myceliophthora thermophila, Paecilomyces* sp. and *Rhizomucor pusillus* were isolated from digested wastewater sludges by Czikkely and Balint (2016). Ahirwar et al. (2017) isolated 68 thermophilic and thermotolerant fungi including *Absidia corymbifera, Aspergillus fumigatus, Chaetomium thermophile, Emericella nidulans, Humicola insolens, Malbranchea cinnamomea, Melanocarpus albomyces, Paecilomyces* sp.*, Scytalidium thermophilum, Sporotrichum thermophile, Thermoascus aurantiacus, Thermomyces lanuginosus* and others on the basis of their potential for growing at 45 °C from various self-heated habitats such as compost, woodchips, wheat straw, bird nesting materials, storage seeds, soil, decaying organic material and litter. Details of occurrence of thermophilic fungi in different ecosystems are given in Table 7.2.

7.2.1 Culturable and Non-culturable Thermophilic and Thermotolerant Fungi

The most critical ecological factor that influences distribution and activities of microbes is temperature (Maheshwari et al. 2000; Johri et al. 1999). Archaea and Eubacteria are the domains to which most of the described thermophilic microorganisms belong (Barns et al. 1996), although 62 °C has been recorded as the maximum temperature limit for Eukaryota (Tansey and Brock 1972). In 1997, Mouchacca reported that more than 50 species of thermophilic fungi grow potentially at relatively elevated temperatures. Johri et al. (1999) found that thermophilic fungi occur in areas where decomposition of organic matter occurs and can be isolated from compost, beach sands, manure, effluents of nuclear reactor, industrial coal mine soils, Saudi Arabian desert soils and Dead Sea valley. Depending on nutritional availability and favourable environmental conditions, these fungi may occur either as active mycelia or as resting propagules. Apart from the above, thermophilic fungi have also been found in some of non-thermogenic areas such as moist and coal soils in Antarctic and sub-Antarctic and Australian soils (Johri et al. 1999) and also in dust on sparingly used library books and aquatic accumulations where temperature at base never passes 6 or 7 °C (Subrahmanyam 1999). For the first time, *Chaetomium senegalense* and *Myceliophthora fergusii* were isolated from temperate soils of North India (Salar and Aneja 2006) and further explained by their biogeography and taxonomy (Salar and Aneja 2007). The diversification of thermophilic fungi isolated from compost samples collected from distinct parts of Amritsar (Punjab, India) was studied by Sharma et al. (2008). For identification and comparison, they used molecular markers such as D1/D2

Table 7.2 Occurrence of thermophilic fungi in different ecosystems

Fungus	Habitat	Thermophile/ thermotolerant	Optimum temperature °C	Optimum pH	Medium	References
Deuteromyces	Jute cuttings	Thermophilic	55	6.0	Mandel's medium	Gomes et al. (1992)
Melanocarpus albomyces	Compost	Thermophilic	40	–	–	Prabhu and Maheshwari (1999)
Lichtheimia ramosa H71D	Sugarcane bagasse compost	Thermotolerant	37	4.5–7.5	PDA	Alvarez-Zuniga et al. (2017)
Aspergillus fumigatus	Soil from rhizosphere, garden compost, Herbivore dung, decomposting bagasse piles, textile wastes, wheat straw and rice straw heaps	Thermophilic	50	–	PDA	Javed et al. (2011)
Aspergillus fumigatus and Aspergillus niger, Canariomyces notabilis, Chaetomium nigricolor and Chaetomium strumarium	Soil of Israeli deserts and Northern territories	Thermotolerant	40	7.2–8.85	Malt extract Agar and Czapek's Agar medium	Grishkan (2018)
Humicola strain	Faeces of llama	Thermophilic	40	6.0	Oatmeal baby food (Quaker) medium	Souza et al. (2010)
Scytalidium thermophilum	Mushroom compost	Thermophilic	45	–	Yeast-glucose agar medium	Straatsma et al. (1994)
Malbranchea cinnamomea NFCCI 3724, *Melanocarpus albomyces, Myceliophthora thermophile* NFCCI 3725	Compost, storage seed soil	Both	45	–	Yeast extract soluble starch agar (YpSs) medium	Ahirwar et al. (2017)

(continued)

Table 7.2 (continued)

Fungus	Habitat	Thermophile/ thermotolerant	Optimum temperature °C	Optimum pH	Medium	References
Rhizomucor pusillus (Lindt) Schipper, *Rhizopus microsporus* van Tieghem, *Aspergillus fumigatus* Fresenius	Soil and coconut residues	Both	Up to 50	5.6	Medium composed of coconut copra flour, agar, chloramphenicol	Córdova et al. (2003)
Malbranchea cinnamomea, *Thermomyces lanuginosus*, *Scytalidium thermophilum*, *Myceliophthora thermophila*, *Paecilomyces* sp., *Thermoascus aurantiacus*, *Myriococcum thermophilum* and *Rhizomucor pusillus*	Digested wastewater sludge	Thermophilic	65–68	–	Potato-glucose agar, Malate-extract agar, Martin-type agar and the microcrystalline cellulose agar	Czikkely and Bálint (2016)
Chaetomium thermophilum, *Humicola insolens*, *Melanocarpus* sp., *Malbranchea* sp., *Thermoascus aurantiacus*		Thermophilic	45–50	7.0	YpSs	Ghatora et al. (2006)
Talaromyces thermophilus strain YM3–4	Tengchong hot springs,	Thermophilic	45	–	PDA	Guo et al. (2011)
Humicola sp.	Horse dung manure	Thermophilic	45	–	YpSs	Kumar et al. (2014)
Myriococcum thermophilum, *Thermoascus aurantiacus*, *and Thermomyces lanuginosus*	Compost	Thermophilic	45–50	–	YpSs	Lee et al. (2014)

Aspergillus fumigatus and Rhizopus sp.	Rice storage products (poha murah)	Thermophilic	45		YpSs	Mene and Naz (2016)
Aspergillus, Thermomyces, Myceliophthora, Thermomucor and *Candida*	Soil, decaying organic matter and sugarcane piles	Both	45	5.0	Medium composed of $FeSO_4$ $7H_2O$, $MnSO_4.H_2O$, $ZnSO_4$ $7H_2O$, $CoCl_2$	Moretti et al. (2012)

(hypervariable regions of 26S rDNA), ITS (internal transcribed spacer sequence) and 18S rDNA regions. A study on species diversity and number of thermophilic fungi in 38 nests of nine species of wetland birds was conducted by Kornillowicz-Kowalska and Kitowski (2013). They found *Aspergillus fumigatus* as a superior species occupying 95% of the nests, and the fungal population was expanded with increase in nest's moisture content. Souza et al. (2014) reported that *Scytalidium thermophilum, Thermomyces ibadanensis* and *Thermomyces lanuginosus* were the main copious species in the second stage of composting to cultivate *Agaricus subrufescens*; rep-PCR was used for characterization of 20 isolates which revealed significant genetic diversity among them, while analysis by DGGE revealed low diversity. Throughout the process of composting, *Scytalidium thermophilum* was reported as the predominant species. In Tengchong Rehai (National Park, China), geothermal sites near alkaline and neutral spring, the diversity of thermophilic fungi was explored by Pan et al. (2010). The fungal isolates were identified based on ITS sequencing; *Scytalidium thermophilum* (syn. *Torula thermophila*) and *T. lanuginosus* were the most predomiant species among them.

7.2.2 Taxonomy

In every ecosystem, there are abiotic and biotic factors making it different and they also have unique characteristics that set them apart like high temperatures and pH, high salt concentrations and/or fusion of these factors. Therefore, as a result of these different environmental conditions, some of microorganisms have evolved transformation which empowers them to survive and colonize in extreme situation. The thermophiles are found in all domains such as Eukarya, Archaea and Bacteria. However, the level of tolerance to increased temperatures is low in Eukarya than in Prokarya, and no species have been observed to grow above 62 °C. The details of taxonomic rank of thermophilic fungi with their cardinal temperatures are provided in Table 7.4. In eukaryotes, some fungi have the potential to propagate and survive actively at high temperatures, but this system of classification is somewhat practical and not suitable for all fungi, as *Aspergillus fumigatus* can grow below 20 °C and also at 50 °C (Mouchacca 2000). Some researchers have proposed that thermophilic fungi originate from ancestors of mesophilic fungi associated with nests of birds, which are found in Australia (Megapodiidae) [Rajasekaran and Maheshwari 1993; Cooney and Emerson 1964]. The birds used decaying plant materials and regulated temperature in their nests, which work like a natural system for composting. Fungi play a main role in decomposition of such substrates by increasing the temperature of the nest to 45 °C (approximately) (Seymour and Bradford 1992). However, they constitute an ecologically defined class; thermophilic fungi are spread in various taxonomic classes, and characteristic species can be found in the phyla Zygomycota, which is now considered as a multiplex of various unrelated subphyla (Hibbett et al. 2007), and Ascomycota (Salar and Aneja 2007). Morgenstern et al. (2012) studied phylogenetics and reported that the fungi that can tolerate heat form a paraphyletic category, which occurs in various phylogenetic lineages in fungal life; this means thermophily had several self-made origins in the Kingdom Fungi. On the other side,

in the Family Chaetomiaceae, which constitutes many heat-tolerant *Ascomycete* fungal species, the most niggardly scenario is that when the thermophilic fungi developed from a common ancestor (van Noort et al. 2013; Morgenstern et al. 2012). According to documented studies, thermophilic fungi have been reported in various culture-based analyses in various habitats such as various types of soil (Powell et al. 2012; Pan et al. 2010; Salar and Aneja 2006), cooling system of power plants, sediments of ponds (Ellis 1980), heap of plant residues (Tansey 1971) and composite systems. The atmosphere, humidity and temperature of these habitats are the suitable elements for expansion of these fungi (Salar and Aneja 2007). Despite their broad-range distribution, these fungi were not active in certain habitats, and it was assumed that it was due to their occurrence as propagules that are basically transported by air (Le Goff et al. 2010; Rajasekaran and Maheshwari 1993). This dispersion takes place majorly from composting systems because of aerosols (Le Goff et al. 2010).

While a few fungi were considered as true thermophiles on the basis of Cooney and Emerson's (1964) concept, many other have been recognized by various names (Mouchacca 1997, 2000). Uncertain taxonomy of thermophilic fungi has led to misidentification and conflicts, and various thermotolerant fungi in applied research are categorized as thermophiles (Mouchacca 2000). The operation of optimum and cardinal growth temperature as a standard for distinguishing thermophilic fungi from thermotolerant fungi is difficult because of the presence of various fungi which do not fit into these groups. To differentiate between thermotolerant and thermophilic strains, there is a possible alternative which classifies fungi on the basis of optimal growth within the range of 40–50 °C and failure to grow at less than 20 °C. In this system, those fungi should be classified as thermotolerant, which have an optimal growth ranging from 40 to 50 °C and can also grow below 20 °C. The correct classification of fungi as thermotolerant and thermophilic is of significant economic and academic interest as several of these fungi are used in biotechnological operation (Mouchacca 2000). Some investigations have been conducted related to diversity and taxonomy of fungi resistant to heat and on the applied part of thermophilic fungi; different synonyms are frequently used to discuss the same fungus. The current taxonomic status of thermophilic fungi is a problem when one compares their results with those of others because distinct systems for nomenclature are applied by the researchers. There is an attractive tool which helps in standardizing fungal nomenclature known as *Index Fungorum* (www.indexfungorum.org), a database for nomenclature in which we can find the latest names of fungi, i.e. basionyms and synonyms, and other information related to their categorization. In addition, the *fungal names* (www.fungalinfo.im.ac.cn/fungalname/fungalname.html) and MycoBank (www.mycobank.org) are important databases in which updated and recent names are registered along with description and illustrations of every taxon. All the above-mentioned databases are the latest repositories for fungal names, for example, for species and genera, and comprising novel fungal names. Currently, a significant step was taken for standardizing biological names of fungal taxonomy. In Amsterdam in a symposium on April 19 and 20, 2011, mycologists established *The Amsterdam Declaration on Fungal Nomenclature*; its aim is to combine the nomenclatural method for sexual and asexual fungi. It aims at abolishing dual existing

nomenclature system for pleomorphic fungi, in which species is explained from distinct phases of reproductive cycle, anamorphic (asexual) or telomorphic (sexual) and acquired separate names (Hawksworth et al. 2011). In 2011, in the 23rd International Botanical Congress in Melbourne, the nomenclature group ratified modifications suggested by mycologists, leading to the new International Code of Nomenclature for Plants, Fungi and Algae (ICN, McNeill et al. 2012). With the evolution of this code, *One fungus = One name* movement was started in order to fix up the fungal nomenclature (Wingfield et al. 2012; Taylor 2011). Since 2012, the changes have been implemented. For various reported fungi, it has not been resolved with regard to which name should be applied under this new rule. On the basis of various criteria, the names are changed, for example, preference is frequently given to the oldest name. For universal well-known species, the latest name of the species, teleomorph or anamorph may be considered. In case the anamorph name is specified, it is required to undergo the agreement made by the General Committee (GC) of the ICN, even if it is the oldest name of the species (Hibbett and Taylor 2013). These modifications in nomenclature of fungi have had impact on fungal taxonomy, including thermophilic fungi. Since 2000, a number of fungal names have undergone through taxonomic changes such as *Stilbella thermophila* was reclassified as *Remersonia thermophila* (Seifert et al. 1997). Some of the fungi, representatives of the genera *Dactylomyces* and *Coonemeria,* have also undergone nomenclature changes and recently categorized as *Thermoascus;* more recently, the genus *Talaromyces* has also been changed. On discussing this genus, it was found that according to phylogenetic analysis, considering multiple molecular markers, this genus does not have thermophilic species (Houbraken et al. 2012). It was observed that *Talaromyces thermophilus* was phylogenetically nearly associated with the type species of *Talaromyces thermophilus,* showing a need to remake the taxonomy of this group. Eventually, this law was approved, and this species was reclassified as *Talaromyces dupontii* (Houbraken et al. 2014); another three species earlier grouped as *Talaromyces* and two species of *Geomithia* have been shifted to the new genus *Rasamsonia,* which involves both thermotolerant and thermophilic fungi (Houbraken et al. 2012). By this approach, it was concluded by the authors that thermophily is confined to species of the genera *Rasamsonia, Thermoascus* and *Thermomyces* within the Eurotiales. The species of *Corynascus* that are identified by some to be thermophilic were reclassified as related to the genus *Myceliophthora,* and a new species of *Myceliophthora guttulata* was isolated from soil in China. That is why this genus has seven truly thermophilic species, namely, *M. fusca, M. fergusii, M. guttulata, M. thermophila, M. sulphurea, M. hinnulea and M. heterothallica* (Zhang et al. 2014; van den Brink et al. 2012). Table 7.3 indicates the nomenclature novelties and changes in name since Mouchacca reported in 2000 (Mouchacca 2000) in taxonomy of thermophilic fungi. Alvarez-Zuniga et al. (2017) reported taxonomic identification of thermotolerant as well as the fast-growing fungus *Lichtheimia ramosa* H71D. This zygomycete fungus was isolated from sugarcane bagasse compost and identified with the help of phylogenetic study on the basis of DNA sequence of ITS (internal transcribed spacer) and subsequent secondary structure examination of ITS2.

Table 7.3 Nomenclature novelties and changes in names since Mouchacca (2000) in taxonomy of thermophilic fungi (Oliveira et al. 2014)

Old name	Current name	MycoBank accession #	References
Talaromyces byssochlamydoides	*Rasamsonia byssochlamydoides*	519,877	Houbraken and Samson (2011)
Talaromyces emersonii	*Rasamsonia emersonii*	519,874	Houbraken and Samson (2011)
Corynascus thermophilus	*Myceliophthora fergusii*	317,954	van den Brink et al. (2012)
Corynascus thermophilus	*Myceliophthora guttulata*	802,335	Zhang et al. (2014)
Corynascus heterothallicus	*Myceliophthora heterothallica*	519,538	van den Brink et al. (2012)
Talaromyces thermophilus	*Thermomyces dupontii*	805,186	Houbraken et al. (2014)

7.2.3 Physiology and Biochemistry of Heat Tolerance

Mesophiles are the major group among fungi that grow better between 5 and 40 °C, and there are a few species that grow above 50 °C, known as thermotolerants and thermophiles, but some of the thermophilic fungi show growth at room temperature, i.e. 20–30 °C; that is why Maheshwari et al. (2000) recommended to redefine thermophilic fungi on the basis of optimal temperature for growth and development at 45 °C and/or above. In eukaryotes, fungi were the only organisms that have a higher temperature range for their growth at 60 °C (Tansey and Brock 1972). The thermophilic fungi can tolerate and grow at pH ranging between 4.0 and 8.0. Rosenberg (1975) reported that the hay with a pH of about 6.5 was colonized with *Rhizomucor pusillus* and *Rhizomucor miehei,* followed by *Malbranchea cinnamomea* and *Thermomyces lanuginosus*, and *Thermomyces thermophilus* having optimal pH near neutral, and *T. emersonii* and *Allescheria terrestris* have been reported to grow in acidic conditions on sugarcane bagasse at pH 3.4–6.0. Thermophilic fungi can be grown on simple media with nitrogen and carbon sources with mineral salts, and it was shown that thermophile fungi do not require any special growth medium; therefore, they are autotrophic for all vitamins (Satyanarayana and Johri 1984; Cooney and Emerson 1964). Several thermophilic fungi have been reported to survive on cellulose, hemicellulose, lignin, pectin and starch (Satyanarayana and Johri 1984). The use of amino acids by thermophilic fungi was studied by Meyer and Armstrong (1970), and it was observed that *P. duponti* and *T. lanuginosus* used single amino acid as a source of nitrogen for growth and did not require any other additional growth factors. Similarly, asparagine was reported to support the sporulation and vegetative growth of *T. lanuginosus* (Maheshwari et al. 2000). *Thermoascus aurantiacus* was reported to grow well on mannitol, dulcitol, citric acid and oxalic acid, whereas *S. thermophilum* showed no growth on dulcitol

Table 7.4 Taxonomic status and cardinal temperatures of thermophilic fungi (Maheshwari et al. 2000)

Current nomenclature	Other names	Optimal temperature °C (T_{opt})	Maximum temperature °C (T_{max})
Canariomyces thermophila Guarro & Samson	–	45	
Chaetomium mesopotamicum Abdullah & Zora	–	45	52
Chaetomium thermophile La Touche	*C. thermophilum*, *C. thermophilium*	45–55	58–61
Coonemeria aegyptiaca (Ueda & Udagawa) Mouchacca	*Thermoascus aegyptiacus*, *Paecilomyces aegyptiaca*	40	55
Coonemeria crustacea (Apinis & Chesters) Mouchacca	*Thermoascus crustaceus*, *Dactylomyces crustaceus*, *Paecilomyces crustaceus*	40	<60
Coonemeria verrucosa (Yaguchi, Someya & Udagawa) Mouchacca	*Thermoascus crustaceus*	30–40	55
Corynascus thermophilus (Fergus & Sinden) van Klopotek	*Thielavia thermophila*, *Myceliophthora fergusii*, *Chrysosporium fergusii*	50	60
Dactylomyces thermophilus Sopp	*Thermoascus thermophilus*, *Thermoascus aurantiacus* (misapplied name)	40–45	
Malbranchea cinnamomea (Libert) van Oorschot & deHoog	*Trichothecium cinnamomeum*, *Thermoidium sulphureum*, *Malbranchea pulchella* var. *sulfurea*	45	57
Melanocarpus albomyces (Cooney & Emerson) von Arx	*Myriococcum albomyces*, *Thielavia albomyces*	45	57
Melanocarpus thermophilus (Abdullah & Al-Bader) Guarro, Abdullah & Al-Bader	*Thielavia minuta* var. *thermophila*	35	50
Myceliophthora hinnulea Awao & Udagawa	–	40–45	>50
Myceliophthora thermophila (Apinis) van Oorschot	*Sporotrichum thermophilum/thermophile*, *Chrysosporium thermophilum*, *Myceliophthoraindica*, *Corynascus heterothallicus*	45–50	55
Myriococcum thermophilum (Fergus) van der Aa 45	–	45	53
Paecilomyces variotii Bainier	–	50	55

(continued)

Table 7.4 (continued)

Current nomenclature	Other names	Optimal temperature °C (T_{opt})	Maximum temperature °C (T_{max})
Rhizomucor miehei (Cooney & Emerson) Schipper	*Mucor miehei*	35–45	57
Rhizomucor pusillus (Lindt) Schipper	*Mucor pusillus*	35–45	55
Scytalidium thermophilum (Cooney & Emerson) Austwick	*Torula thermophila*, *Humicola grisea* var. *thermoidea*, *Humicola insolens*	40	58
Stilbella thermophila Fergus	–	35–50	55
Talaromyces byssochlamydioides Stolk & Samson	*Paecilomyces byssochlamydioides*	40–45	>50
Talaromyces emersonii	*Geosmithia emersonii*, *Talaromyces duponti* and *Penicillium duponti* (misapplied names)	40–45	55
Talaromyces thermophilus	*Penicillium duponti*	45–50	60
Thermoascus aurantiacus	*Thermoascus aurantiacus* sensu Cooney & Emerson (misapplied name)	49–52	61
Thermomyces ibadanensis Apinis & Eggins	–	42–47	61
Thermomyces lanuginosus Tsiklinskaya	*Humicola lanuginosa*	45–50	60
Thermomyces stellatus (Bunce) Apinis	*Humicola stellata*	40	50
Thielavia australiensis Tansey & jack	–	35–40	50
Thielavia pingtungia Chen K.-Y. & Chen Z.-C.	–	40	>50
Thielavia terrestris (Apinis) Malloch & Cain	*Allescheria terrestris*, *Acremonium alabamensis*	40–45	52

but poorly grew on citric acid and oxalic acid (Subrahmanyam 1977). The growth of thermophilic fungi was supported by simple sources of nitrogen, for example, asparagine and nitrates of potassium and sodium (Satyanarayana and Johri 1984; Subrahmanyam 1977). Urea was reported to support the growth of *Acremonium alabamensis*, *Rhizopus microsporus*, *Thermomyces lanuginosus* (Satyanarayana and Johri 1984), *S. thermophilum* and *T. aurantiacus* (Subrahmanyam 1980, 1997).

The thermophilic fungi require succinate in place of glucose and ammonium sulphate for their growth as succinate stimulated the growth because of its buffering action (Wali et al. 1978) The simultaneous utilization of sucrose and glucose by

P. duponti and *T. lanuginosus* at 50 °C was reported by Maheshwari et al. (1988), and the mycelium yields of *Thermomyces lanuginosus* in mixed and single carbon sources were almost the same. When it comes to oxygen requirement, thermophilic fungi needed a high amount of oxygen for sporulation as compared to that for growth (Johri et al. 1999). Most fungi for their trace growth require 0.2% oxygen, while for moderate growth, they require 0.7–1.05% and 1.0% for their sporulation (Sharma and Johri 1992). In fungi, the termination of growth was attributed to the generation of degenerative factors, change of pH, exhaustion of nutrients and more. In static cultures, exhaustion of oxygen was found to be accountable for halting growth of *T. lanuginosus* (Prasad and Maheshwari 1978). Mannitol was synthesized by *T. lanuginosus* when cultivated at 30–50 °C, while trehalose was produced and profusely stored in mycelia, and it produced spores at 50 °C which indicated that accumulation of trehalose is possibly a temperature-associated phenomenon (Johri et al. 1999). In thermophilic fungi, spermidine, spermine and putrescine were widely present (Singhania et al. 1991). In growing mycelium of *T. lanuginosus*, the level of free polyamines was higher than that in old steady-phase mycelium and the amount of polyamine decreased at temperatures below or above optimal growth temperatures (45 °C). Difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase (ODC), greatly inhibited the growth of mycelia of *Rhizomucor pusillus, Talaromyces emersonii* and *Thermomyces lanuginosus*, which suggested the presence of the ODC pathway in these fungi. Similarly, difluoromethylarginine (DFMA), an inhibitor of arginine decarboxylase (ADC), reduced the mycelial growth considerably, and possibly, this could be due to either transformation of DFMA to DFMO by arginase or due to the presence of ADC (Singhania et al. 1991).

In general, under anaerobic conditions, thermophilic fungi fail to grow. Under anaerobic conditions, respiration and growth of *Thermoascus aurantiacus* was critically affected (Noack 1920). As compared to aerobic conditions, *Scytalidium thermophilum* grew better under anaerobic or microaerophilic conditions at elevated temperatures. An attractive morphogenetic effect of anaerobiosis was recorded in *Talaromyces duponti* in which the fungus creates only a single conidial stage under aerobic conditions while a sexual stage under anaerobic conditions (Cooney and Emerson 1964). Temperature is a critical factor influencing the development and growth of thermophilic fungi (Rosenberg 1975). In thermophilic fungi, a sexual and asexual reproductive phase occurs in a limited temperature range compared to the vegetative growth, and in this way, they act like mesophiles. Vegetative growth occurs at 50 °C, while optimal sporulation occurs at 40 °C (Prasad and Maheshwari 1978). Both *Thermoascus aurantiacus* and *Chaetomium thermophile* produced ascocarps at 45–50 °C (Tansey and Brock 1972). It was found that most of the thermophiles can survive on exposure to 60 °C for 1–48 hours. *T. lanuginosus* and *T. aurantiacus* were reported to survive on exposure to 72 °C for 2–10 minutes (Satyanarayana and Johri 1984), and in *T. aurantiacus* and *R. pusillus,* respiration rates were found to be high at 45 °C (Johri et al. 1999).

In phospholipids, the increased quantity of saturated fatty acids at increased temperatures and unsaturated fatty acids at decreased temperatures is called as homeoviscous adaptation. In *T. thermophiles,* the degree of unsaturation remained

unchanged after temperature changes from 50 to 33 °C (Wright et al. 1983). In contrast, in phospholipids, the amount of linoleic acid (18:2) was twofold higher at 30 °C than at 50 °C (Rajasekaran and Maheshwari 1993). The lack of ability to maintain membrane fluidity may not be a cause for their growth at elevated temperatures (Maheshwari et al. 2000). In thermophilic fungi, the high turnover of proteins/enzymes has been considered as one of the reasons for their existence at elevated temperatures, and this rapid turnover hypothesis is considered as dynamic hypothesis of thermophily. In thermophilic fungi such as *R. pusillus*, *M. cinnamomea, and P. dupontii* and mesophilic fungi such as *P. chrysogenum* and *P. notatum*, the breakdown rates of protein, especially the pulse-labelled proteins, were investigated by Miller et al. (1974); on observation, they found that the developing cells of thermophiles and mesophiles exhibited slight breakdown of protein, but the non-growing cells showed a similar rate of protein breakdown. In thermophilic fungi, the fraction of soluble protein breakdown was twice than that of mesophilic fungi, indicating the improved yield of soluble protein which is significant for existence of thermophilic fungi at elevated temperatures.

In thermophiles, high protein turnover may disturb their growth rate as compared to that in mesophiles. Extracellular enzymes like cellulases and xylanases (Kaur et al. 2004), phytases (Singh and Satyanarayana 2009a, b) and glucoamylases (Kumar and Satyanarayana 2003) from thermophilic fungi are glycoproteins with 13–68% of carbohydrate, which plays a key role in stabilizing the enzymes' conformation at higher temperatures. In the RNA of *T. lanuginosus*, an increased number of ribose methylation has been noticed as compared to that in any other non-vertebrates (Nageswara Rao and Cherayil 1979); but the significance of this observation has not been understood. In thermophilic fungi, the formation of heat shock proteins (HSPs) was reported by Trent et al. (1994). In *T. lanuginosus*, the conidia mature at 50 °C after 60 minutes of heat shock treatment at 55 °C, showing improved survival in comparison to non-heat-shocked conidia.

7.2.4 Genomics

The complete sequences of genomes of thermophilic ascomycetes, *Thielavia terrestris* and *Myceliophthora thermophila*, have been published (Berka et al. 2011). The genome of *T. terrestris* is of 36.9 Mb in six chromosomes with 9813 protein-coding genes, while the genome of *M. thermophila* is of 38.7 Mb in seven telomere-to-telomere chromosomes with 9110 protein-coding sequences. In both species, more than 200 genes encode carbohydrate-active proteins (CAZymes) like polysaccharide lyases (PLs), glycoside hydrolases (GHs) and carbohydrate-binding modules. The secretomes of *M. thermophila* and *T. terrestris* comprise 683 and 789 proteins, respectively, and nearly, only one-third of these proteins are equivalent to CAZymes. Alfalfa straw hydrolysis revealed that the mixture of enzymes from both fungi led to release of increased amounts of reducing sugars in comparison with the mesophilic fungi *T. reesei* and *C. globosum*. *Thielavia terrestris* and *Myceliophthora thermophila* genomes possess high GC content in coding regions

in comparison to mesophilic species; G:C pairs are additionally strong to thermal denaturation, and this high G:C level may be a modification in genes encoding protein at higher temperatures (Berka et al. 2011). The proteomics and genomics of three other species of the genus *Myceliophthora* (*M. fergusii, M. heterothallica* and *M. hinnulea*) have also been studied by van den Brink et al. (2013), but the potential of plant biomass degradation was high in *M. heterothallica* compared to that of other two species; the isolates of *M. hinnulea,* however, grew gradually on biomass of plants. Most of the isolates displayed improved growth when the amount of xylan was more in plant biomass. The isolates of *Myceliophthra thermophila* CBS 669.85 and CBS 866.85 had rapid growth rate on xylan-poor and cellulose-rich source; in mesophiles, the activities of enzymes were optimum at 50 °C, and for thermophilic *Myceliophthora* isolates, it was 70 °C. Sexual breeding among the incompatible mating-type *M. heterothallica* strains CBS 203.75 and CBS 663.74 was also analysed by authors, which yielded the hybrid progenies FP 711.14 and FP 711.02 showing increased cellobiohydrolase activity in contrast to parental strains. The complete genome of *Chaetomium thermophilum*, found in decomposed plant biomass or dung and in soil, was sequenced by Amlacher et al. (2011). The size of its genome was 28.3 Mb and was fractionated into eight chromosomes plus 7227 predicted protein-encoding genes. van Noort et al. (2013) carried out a systematic study of genetic variation among mesophilic and thermophilic fungi using *C. thermophilum*, *T. heterothallica* and *T. terrestris,* and their proteomes and genomes were analysed for describing the evolution and molecular basis of thermophily. The thermophilic fungi have significantly smaller genomes than their mesophilic counterparts like *Neurospora crassa* and *C. globosum*. The small size of the genome was due to lesser protein-coding sequences and smaller intergenic and intron regions, and the genome also showed barely transposable element-associated sequences indicating that the transposition was not an adaptation to thermophily. The genomes of thermophilic fungi interestingly possess fewer genes which encode enzymes for degrading complex carbohydrates. Three cellobiose dehydrogenases are encoded by the genome of *C. thermophilum*, while the genome of *N. crassa* and *C. globosum* encodes only two cellobiose dehydrogenases, which showed that the former has higher cellulolytic capacity. Melanin production protects the cells of fungi from drought, increased temperatures and UV radiation; this appears to represent transformation to thermophily in *Thielavia heterothallica* and for scytalone dehydratase (key enzyme for melanin production); this species presents three orthologs. Thermophilic fungi synthesize increased levels of tryptophan, proline and arginine but lower levels of lysine and aspartic acid than mesophilic fungi. The proteins of *Chaetomium thermophilum* contain high cysteine; this may represent an adaptation to thermophily. van Noort et al. (2013) had also analysed (not yet published) the amino acid sequence of proteins of *T. thermophilus* and *T. lanuginosus.* A significant depletion of glycines and enrichment in alanine and arginine had been recorded in *T. heterothallica*, *T. terrestris* and *C. thermophilum*. The researchers observed an expanded mutational bias between prolines and small amino acids in thermophile genomes. Prolines make protein configuration extra stringent and rare to unfold. There are alternative mutations in thermophiles like from threonine to alanine, aspartic acid to glutamic acid and lysine to arginine. In

thermophilic as well as mesophilic fungi, the global GC content did not vary remarkably. Based on bioinformatic analysis, the researchers predicted 38,385 adaptive mutations in 2064 single copy of proteins, and out of them, some were analytically proved through protein engineering. The draft genome of *Thermomyces lanuginosus* SSBP isolated from South Africa soil has been reported as most potent producer of xylanase till date by Mchunu et al. (2013); the size of its genome was 23.3 Mb with 5105 genes and GC content of 52.14%, but this level may increase to 55.6% if only coding regions are contemplated. The predicted CAZymes were estimated to 224 proteins. *Rhizomucor miehei*, utilized as a source of industrial proteases and lipases, grows at 50 °C or above. Zhou et al. (2014) recently sequenced the transcriptome and genome of *R. miehei* CAU432 isolated from self-heating fodder in China; the size of its genome was 27.6 Mb having 10,345 protein-coding genes, accounting for 47.1% of genome. On average, 4.5 introns/gene are present; introns are made of 90 base pairs, and 10 chromosomes ranging from 0.55 to 5.8 Mb are revealed by PFGE (pulsed-field gel electrophoresis); the standard GC content, 43.8%, was less than that for *M. thermophila* (51.4%), *T. lanuginosus* (52.14%) and *T. terrestris* (54.7%) (Berka et al. 2011; Mchunu et al. 2013; van Noort et al. 2013), but it was higher than that of the average value (51.4%) of mesophilic zygomycetes. When the genome of mesophilic *R. oryzae* (Ma et al. 2009) was compared with *R. miehei* genome, it was found that the genome of the latter has tenfold less repetitive sequences. Multigenic families for chitin and starch degradation have been explained. The relative RNA-Seq analysis of *Rhizomucor miehei* produced at two varied temperatures showed that at 30 °C, 190 genes were exclusively expressed, while at 50 °C, 128 genes were transcribed and about 2120 genes were distinctly expressed with more than twofold changes. About 849 genes were upregulated, and many of them were associated with protein folding as well as protein synthesis, while around 1268 genes were downregulated involving those included in the degradation of proteins by proteasomes. The gene products that are associated with metabolism of thermo-stabilizers, non-reducing disaccharides and trehalose were also expressed at 50 °C. Fujii et al. (2015) sequenced the genome of *Talaromyces cellulolyticus* Y-94 (formerly *Acremonium cellulolyticus*) which produces a high titre of cellulase. Its genome is of 36.4 Mb and has genes for various enzymes useful in lignocellulosic biomass decomposition such as amylases, pectinases, cellulases and hemicellulases. The Genozyme Research Project (http://fungalgenomics.ca/wiki/ Fungalgenomes) at that moment was sequencing the genomes of thermophiles such as *Chaetomium olivicolor* CBS 102434, *Acremonium thermophilum* ATTCC 24622, *Myceliophthora hinnulea* ATCC 52474, *Remersonia thermophila* ATCC 22073 (syn. *Stilbella thermophila*), *Humicola hyalothermophila* CBS 454.80, *Dactylomyces thermophilus* ATCC 26413, *Talaromyces emersonii* NRRL 3221 and *R. miehei* CBS 182.67, and additionally, it also planned to sequence *Thermophymatospora fibuligera* ATCC 62942, *Thermoascus aegyptiacus* ATCC 56490 and *Melanocarpus albomyces* ATCC 16460; the publication of such results will make an unprecedented contribution to thermophily in the fungal kingdom and their utilization in industries.

7.2.5 Role in Nature (Organic Matter Decomposition)

The activity of thermophilic fungi in natural combustion of hay was first investigated by Miehe (1907). He used an insulated chamber and observed that within a few days moist leaves/hay get self-heated to 60–70 °C, whereas steamed hay did not get self-heated. But the steamed hay got heated up when it was mixed with soil particles or washing from unheated fodder or added to pure cultures obtained from self-heated fodder; on the basis of these findings, Miehe believed that heating of plant organic material to 60–70 °C was due to natural and biotic activities of thermophilic fungi and other microorganisms in that heated area. The factors that influenced microbial thermogenesis, oxygen availability, nutrient supply and moisture level were of prime importance. Cooney and Emerson (1964) reported that for the decomposition of complex organic residues of plants, fungi were well furnished with all essential enzymes. Satyanarayana et al. (1977) and Tansey (1973) had also reported composting of plant material in nests of animals and birds, and decomposed residues of plants have been observed to be full of thermophilic fungi. The highest temperature of 68.5 °C was observed on the fifth day in compost (Miehe 1907) while a temperature of 53–47 °C and 67 °C in the centre of paddy and wheat straw composts, respectively. The C:N ratio and size of composting matter affect the decomposition speed, and during the phase of higher temperature, the number of thermophilic microorganisms increased. In compost, some volatile metabolites such as ammonia and others are also produced by thermophilic fungi. The volatile metabolites emitted from *C. thermophile* and *M. thermophila* inhibited the conidial germination of *Thermomycese lanuginosus,* and from the above observations, it was proposed that thermophilic fungi produce volatile metabolites in compost. A wide variety of enzymes were secreted by thermophilic fungi which have varied physicochemical characteristics and functional properties under different environmental conditions (Singh 2016; Singh et al. 2016). Abundance of hemicellulolytic enzymes has been studied from *M. thermophila, C. thermophile, S. thermophilum, T. aurantiacus* and *T. emersonii* (Singh 2016; Singh et al. 2016; Bala and Singh 2016, 2017; Rawat et al. 2005; Thakur et al. 1992). In nature, organic materials are generated in a huge amount and decomposed by microbial activities. On ground surface, organic material decomposed slowly at ambient temperatures, but this process can be accelerated by collecting organic materials into heaps for energy conservation, and this speeds up the activity for decomposition of organic material with the help of mixed microbial population in a moist, aerobic and warm environment. The thermophilic fungi colonize a broad range of forest and agricultural residues such as bagasse, wool, hemp straw, woodchips, wheat and rice straw. Fast growth of thermophilic fungi causes self-heating of organic material which further depends on the existence of soluble organic materials in the substrates. Cellulose, hemicellulose, lignin and starch are some available substrates that are used by fungi (Sharma and Johri 1992; Singh et al. 2016).

7.2.6 Potential Applications of Thermophilic Fungi and their Products

Thermophilic fungi can colonize at extreme ecological niches because they are capable of withstanding high temperatures, and therefore, their enzymes can also remain stable at high temperatures. The thermophilic fungi have been widely used for extracellular thermostable enzymes and protein production, as these fungi can be easily and rapidly grown on low-cost media including agroresidues. The versatile properties of their enzymes make them excellent candidates for multifarious biotechnological applications. Recently, these fungi have been gaining importance for large-scale fermentation in biotechnological industrial processes operated at elevated temperatures (Salar 2018; Shaik 2006). Besides industrial use, these fungi have been studied worldwide for protein engineering and enzyme technology. The proteins/enzymes produced by thermophilic fungi tend to be more thermostable than those produced by mesophilic fungi. Thermophilic fungi have potential to degrade organic matter to produce intra- and extracellular enzymes, antibiotics, amino acids, polysaccharides, phenolic compounds, sterols, SCP and nutritionally enriched feeds, act as natural scavengers and biodecomposers and be a suitable agent for bioconversion, for example, their use in mushroom compost preparation (Singh 2016; Singh et al. 2016). Thermophilic fungi are known as nature-borne biotechnologists. They reduce the risk of contamination caused by mesophilic microbes in biotechnological processes which are conducted at elevated temperatures (Singh et al. 2016). The potential applications and products of some thermophilic fungi are given in Table 7.5 and also discussed below under different headings.

7.3 Thermostable Enzymes

A variety of thermophilic enzymes like lipases, xylanases, proteases, cellulases, amylases, pectinases and phytases were reported by thermophilic fungi and have been used in biotechnological processes such as bio-bleaching of paper and pulp, generation of fermentable sugars for production of biofuel/bioethanol using lignocellulosic residues, clarification and extraction of fruit juice, degumming of natural fibres, refinement of vegetable fibres, wastewater management and curing of tobacco, cocoa, and coffee (Singh et al. 2016). An array of enzymes (high efficiency but low activity compared to mesophilic fungi) with multiple applications is produced by thermophilic fungi (Berka et al. 2011). In self-heating composts, thermophilic fungi were the main components; they can break down cellulose quickly as compared to mesophilic and are prodigious cellulase producers, for example, *Trichoderma reesei* at 40–50 °C (Vafiadi et al. 2009; Kaur et al. 2004). Thermophilic fungi produce plant biomass-degrading enzymes that have optimal temperature between 55 and 70 °C (Vafiadi et al. 2009). Enzymes of thermophilic fungi, like xylanases, have received more importance because of their role in prebleaching of pulp in paper industry which reduced the amount of chlorine for the brightening process in pulp bleaching. An endoglucanse of *Myceliophthora thermophila*

Table 7.5 Potential application of the thermophilic fungi and their products

Environmental aspect	Thermophilic fungi	Substrate	Product(s)	References
Solid waste management	*Chaetomium cellulolyticum*	Agricultural residue	Upgraded feed, SCP, enzymes	Satyanarayana et al. (1992)
	Rhizomucor miehei	–	Protease	Thakur et al. (1993)
	Thermomucor indicae-seudaticae	Wheat bran	Glucoamylase	Kumar and Satyanarayana (2007)
	Sporotrichum thermophile	Sesame oil cake	Phytase	Singh and Satyanarayana (2006)
	Sporotrichum thermophile	Citrus peel	Xylanase, pectinase, cellulase	Kaur and Satyanarayana (2004)
	Thermomyces lanuginosus	Wheat bran	Xylanase	Kamra and Satyanarayana (2004)
	Chaetomium thermophilum, Chaetomium sp., *Malbranchea cinnamomea, Myriococcum thermophilum, Scytalidium thermophilum, Remersonia Thermophila, Thielavia terrestris*	Wheat straw, manure	Mushroom	Straatsma et al. (1994)
	Chaetomium thermophilum, Humicola insolens, Melanocarpus sp., *Malbranchae* sp., *Thermoascus aurantiacus*	Corn cob	Xylanase	Ghatara et al. (2006)
	Aspergillus terreus M11	Corn Stover	Cellulases	
	Aspergillus fumigatus M.7.1 and *Myceliophthora thermophila* M.7.7	Corn straw	Cellulase and xylanase	Moretti et al. (2012)

(continued)

Table 7.5 (continued)

Environmental aspect	Thermophilic fungi	Substrate	Product(s)	References
	Sporotrichum thermophile LAR5	Wheat bran	Cellulase	
	Myceliophthora thermophila JCP 1–4	Lignocellulosic biomass	Endoglucanase, glucosidase, xylanase and avicelase	Pereira et al. (2015)
	Sporotrichum thermophile	Wheat bran and sugarcane bagasse	Phytase	Kumari et al. (2015)
	Sporotrichum thermophile	Cane molasses	Xylanolytic and cellulolytic enzymes	Bala and Singh (2016)
	Sporotrichum thermophile BJAMDU5	Cotton oil cake and wheat straw	Cellulolytic and xylanolytic enzymes	Bala and Singh (2017)
	Sporotrichum thermophile	Cane molasses	Phytase	Singh and Satyanarayana (2006) and (2008)
	Malbranchea cinnamomea NFCCI 3724, *Melanocarpus albomyces*, *Myceliophthora thermophila* NFCCI 3725	Wheat bran	Xylanase and mannanase	Ahirwar et al. (2017)
	Aspergillus fumigatus JCM 10253	Wheat bran	Cellulase and xylanase	Saroj et al. (2018)
Bioremediation	*Talaromyces emersonii*	Polluted water	Uranium	Bengtsson et al. (1995)
	Mucor sp., *Rhizopus* sp.	Polluted water	Heavy metal	Bengtsson et al. (1995)
	Thermomucor indicae-seudaticae	Dye decolourization	Water purification	Taha et al. (2014)
Bioethanol	Thermophilic moulds	Agricultural residue	Sugars, ethanol	Hahn-Hägerdal et al. 2007
	Malbranchea flava	Cotton stalk	Sugars, ethanol	Sharma et al. (2016)
	Aspergillus niger URM 6642	Rice meal	Sugars	Oriente et al. (2015)

expressed in *Aspergillus niger* showed increased rate of hydrolysis for carboxymethylcellulose as compared to the same enzyme from a mesophilic fungi, *T. reesei* (Tambor et al. 2012). Grajek (1987) reported production of increased levels of xylanases from sugar beet pulp under SSF by *H. lanuginosa* and *T. aurantiacus,* and endoxylanase production from wheat straw under SSF was reported by Kalogeris et al. (1998). The production of phytase from sesame oil cake using SSF was reported by Singh and Satyanarayana (2006). Kaur and Satyanayana (2004) reported maximum secretion of cellulolytic, xylanolytic and pectinolytic enzymes by *M. thermophila* in SSF with a combination of citrus peel and wheat bran. Also obtained higher xylanase production on wheat bran in SSF among all tested ligno-cellulosic substrates by *T. lanuginosus*. Cellulose and hemicellulose are the main constituents of plant organic material, for example, rice and wheat straw and sugarcane bagasse, and thermophilic fungi exploit these materials for secretion/ production of a variety of hydrolytic enzymes. Kalogeris et al. (2003) investigated production of extracellular cellulolytic enzymes in SSF by *Thermoascus aurantiacus*. Dos Santos et al. (2003) reported xylanase production from *T. aurantiacus* on sugarcane bagasse in SSF. In nature, after cellulose, the second most abundant polysaccharide is xylan, and its total decomposition required the combined operation of many hydrolytic enzymes such as endo-xylanases (EC 3.2.1.8), which break β-1,4-linked xylose randomly, and β-xylosidases (EC 3.2.1.37), which hydrolyze xylooligomers, and many other side chain splitting enzymes like acetyl xylan, α-arabinosidase and α-glucuronidase, which help in the release of other sugars such as arabinose and glucuronic acid that are attached to the backbone of xylose. Pereira et al. (2015) isolated heat-tolerant *Myceliophthora thermophila* JCP 1–4 from environment, which produced thermophilic endoglucanase, glucosidase, xylanase and avicelase using lignocellulosic biomass as the substrate in SSF. They used the crude enzyme for saccharification of glycerol and microwave-pretreated sugarcane bagasse and reported 2.2 and 1.95 g/l yield of glucose and xylose, respectively. Reported production of phytase (1881.26 U/g DMR) from the thermophilic mould *Sporotrichum thermophile* using mixed substrates, sugarcane bagasse and wheat bran in SSF at 45 °C and also found that the enzyme improved the generation of nutritional components (reducing sugars, soluble protein and inorganic phosphate) from poultry feed at different time intervals. Concomitant production of cellulolytic and xylanolytic enzymes from *Sporotrichum thermophile* in SmF by utilizing cane molasses was reported by Bala and Singh (2016). The effectiveness of the enzyme cocktail in rice straw and waste tea cup paper hydrolysis was determined, and they found that 578.12 and 421.79 mg/g of substrate sugars were produced successfully by the enzyme cocktail from waste tea cup paper and rice straw, respectively, after 24 h. Bala and Singh (2017) reported high titres of thermostable xylanolytic and cellulolytic enzymes from *Sporotrichum thermophile* BJAMDU5 in SSF using a mixture of cotton oil cake and wheat straw (1:1 ratio) after 72 h at 45 °C, and they also found that nutritional properties of bread were improved by supplementation of these enzymes. Singh and Satyanarayana (2006, 2008) reported phytase production in cane molasses

medium from *Sporotrichum thermophile* at 45 °C after 5 days. They observed that the enzyme was resistant to pepsin and trypsin, acid stable and thermostable, and therefore, this enzyme can be used for improving nutritional properties of animal feed in animal feed industry and can check environmental phosphorus pollution. Phytic acid content in dough was efficiently hydrolyzed by this phytase followed by release of soluble phosphate from bread. The bread prepared with this phytase has a low amount of antinutrient (phytic acid). Bajaj et al. (2014) reported cellulase production from *Sporotrichum thermophile* LAR5 by using wheat bran. They utilized this enzyme for saccharification of acid-pretreated rice straw and suggested that this thermophilic cellulase has the potential for transforming lignocellulosic biomass.

7.4 Food and Feed

A large number of biomolecules are produced by *M. thermophila* which have several industrial applications. Fructooligosaccharides and xylooligosaccharides are popular for displaying prebiotic activity and other health-related benefits by assisting lactic acid bacteria (LAB) such as *Lactobacillus* and *Bifidobacterium* sp. in the gut (Sadaf and Khare 2014; Christakopoulos et al. 2003; Katapodis et al. 2003). Besides prebiotics, they are also applied in pharmaceutical, feed and food industries. Katapodis et al. (2003) observed biosynthesis of FOS through in-growth of *M. thermophila* on medium with a higher level of sucrose. Christakopoulos et al. (2003) reported generation of xylooligosaccharides by the activity of *M. thermophila* (family 11) endoxylanase from birchwood. The xylooligosaccharides were active against a variety of Gram-negative and ram-positive aerobically grown *Helicobacter pylori* and bacteria. Sadaf and Khare (2014) reported production of XOs from endoxylanase of *M. thermophila* from xylan hydrolysis at 45 °C and pH 7.0. The existence of xylobiose, xylotrios and xylotetraose detected by HPLC and the level of xylotetraose increased to 80–82% (approximately) in 6 hours, and the lack of xylose in xylooligosaccharides is highly important and beneficial for uses as such in food additives and prebiotics. Several attempts have been made to produce protein-enriched SCP (single-cell protein) and upgraded feeds (Johri et al. 1999). The intake and digestibility of cellulosic feed stuffs can be improved by applying biological treatments. The cellulolytic thermophilic fungi have some advantages over mesophilic fungi such as higher growth rate and cellulose breakdown rate, active over a broad range of temperatures (20–55 °C) and good sources of protein. The residues of forest and agriculture were converted into protein-enriched feed using *C. cellulolyticum* (Chahal et al. 1981).

7.5 Bioethanol

At present, ethanol is one of the most common renewable fuels that is produced from grains (starch) or sugars, and consequently, in the future, universal consumption of ethanol will be mostly dependent on the production by lignocellulosic materials. In this context, the main provocation is optimizing the combination of enzymes, process engineering, metabolic engineering and fermentation technology. In the twenty-first century, one of the biggest challenges is to fulfil the increasing demands of energy for industrial processes and transport; availability of raw materials in a sustainable manner to the industries is other challenge in this field. Worldwide, many countries have formed rules and regulations for the utilization of bioethanol to blend traditional fuels such as petrol. In the USA, Brazil and some European countries, ethanol has already been introduced nationwide, and it can also be used as neat and/or mixed with petrol in dedicated engines, using the benefit of high heat of vaporization and higher octane number with reduced emissions of CO, unburnt hydrocarbons and particulate matter. The enzymes of thermophilic fungi degrade lignocellulosic materials that will play a key role in the lignocellulosic residue saccharification into reducing sugars followed by the fermentation of these sugars by bacteria and yeasts. McClendon et al. (2012) cultivated *T. aurantiacus* on different lignocellulosic substrates for producing glycoside hydrolases. The saccharification of ionic liquid-pretreated *Panicum virgatum* (switch grass) was carried out by using crude culture filtrates. It was observed that enzymes of *T. aurantiacus* released more sugars (glucose) than that of *T. terrestris,* and it was also found that a higher level of activity at elevated temperatures was retained by enzymes of *T. aurantiacus* than the commercial Novozyme (Cellic CTec2) enzyme mixture. Ammonia fibre or ionic liquid and dilute acid-pretreated switch grass was effectively hydrolyzed by *T. aurantiacus* enzymes with concomitant release of reducing sugars. Pereira et al. (2015) reported *M. thermophila* JCP 1–4 as a good producer of endoglucanase, β-glucosidase, xylanase and avicelase with activities of 357.51 U/g, 45.42 U/g, 931.11 U/g and 3.58 U/g, respectively, and these enzymes were stable at 30–60 °C and most active at 55–70 °C. The crude enzymatic extracts were used for saccharification of microwave and glycerol-pretreated sugarcane bagasse, which yielded 15.6% and 35.13% glucose and xylose, respectively. Sharma et al. (2016) reported *Malbranchea flava*, a thermophilic fungus, as potent producer of xylanase and xylan debranching accessory enzymes, and a high ethanol level of 46.0 g/l was attained from acid-pretreated cotton stalks with the addition of *M. flava* xylanase in comparison to 39 g/l without addition of xylanase. Saroj et al. (2018) reported thermophilic *Aspergillus fumigatus* JCM 10253 as a potent extracellular cellulase, β-glucosidase and xylanase producer and also its application in degradation of lignocelluloses. They reported maximum CMCase, FPase, β-glucosidase and xylanase activities of 26.2, 18.2, 0.87 and 2.6 IU/ml, respectively, at 50 °C after 144 hours of incubation. The thermostable crude cellulase had optimal activity at 60 °C, while FPase, β-glucosidase and xylanase exhibited optimal activity at 50 °C, suggesting that these enzymes can be useful in biofuel industries.

7.6 Biotransformation

Thermophilic fungi can be used as a potent source for thermostable enzymes of commercial and scientific interest in synthetic chemistry (Bodai et al. 2003). For the biotransformation of albendazole, an anti-helminthic drug, to generate novel and active metabolites of industrial interest, *Rhizomucor pusillus* was used, and these metabolites include N-methyl metabolite of albendazole sulphoxide, albendazole sulphone and a novel product (Prasad et al. 2011). The expansion of novel biocatalytic methods is an ongoing developing domain of genetic engineering, microbiology and chemistry since these are eco-friendly biocatalysts, easy to handle and selective (Bodai et al. 2003). Considering the increasing demand for chemical compounds with chiral structure, new microorganisms and their enzymes have been subjected to large-scale screening for the production of these chemicals. Bodai et al. (2003) reported 14 thermophilic fungi including *S. thermophilic* CBS-183.64 and CBS-147.64, *C. thermophilum* TUB-F-69, *T. emersonii* NRRL-3221, *M. thermophila* TUB-F-39, *Thermomucor indicae-seudaticae* NRRL-6429, *T. thermophilus* NRRL-2155 and NRRL-5208, *T. lanuginosus* ATTCC-38.905 and CBS-224.63, *Paecilomyces* sp. TUB-F-70 and *T. aurantiacus* TUB-F-43 cultivated in shake flasks displayed carboxylesterase/lipase activities on p-nitrophenyl butyrate, p-nitrophenyl palmitate and olive oil, and enzyme powders of these fungi precipitated by acetone showed a wide range of enantiotopic selectivity such as acetylation in contrast to the most common commercialized enzymes. Hunter et al. (2008) found that the transformation of progesterone, testosterone, dehydroepiandrosterone, steroids and pregnenolone by the thermophilic *Rhizomucor tauricus* was oxidative with allylic hydroxylation of the predominant route of attack which functionalized the skeleton of steroid. A broad-range biocatalytic activity was noticed along with variation at all four rings of steroid nucleus and C-17 side chain, when progesterone, testosterone, androst-4-ene-3,17-dione and 17-acetoxy-5-androstan-3-one were incubated with *M. thermophila* CBS 117.65 (Hunter et al. 2009). This was the first thermophilic fungus that revealed the cleavage of side chain in progesterone and transformation of saturated 17-acetoxy-5-androstan-3-one which yielded 4-hydroxy-3,4-seco-pregn-20-one-3-oic acid formation, and this mould has also been observed to develop reversible oxidation and acetylation of 17-alcohol of testosterone. *T. emersonii* and *Acremonium alabamense* convert cholesterol into cholestenone, and the former convert sitosterol and stigmasterol into stigmostadienone (Satyanarayana and Chavant 1987). Bioconversion of ferulic acid into vanillic acid by *Sporotrichum thermophile* was reported by Topakas et al. The metabolism of ferulic acid by *S. thermophile* evidently takes place through degradation of propenoic chain, and 4-hydroxy-3-methoxystyrene (4-vinylguaiacol) formation was found, which was probably metabolized to vanillic acid, and in addition to these intermediates, guaiacol was identified, typically as a result of non-oxidative decarboxylation of vanillic acid. The quantity of ferulic acid used and the source of carbon on which biomass is grown affect bioconversion of ferulic acid to vanillic acid. Under optimal conditions, production of vanillic acid

from ferulic acid by *S. thermophila* was found to be very high (4798 mg/L) along with a molar yield of 35%. Sreelatha et al. (2018) isolated a thermophilic fungus, *Thermomyces lanuginosus*, with ability to biotransform using spironolactone as a model drug. For this process, they followed a two-stage fermentation protocol; HPLC was used for the identification of transformation of spironolactone, and LC-MS/MS analysis was done for structure elucidation of metabolites. According to their study, *T. lanuginosus* was the potential organism for transformation of spironolactone to four metabolites, namely canrenone, 6β-OH-7α-thiomethylspironolactone, 7α-thiospironolactone and 7α-thiomethylspironolactone (major mammalian metabolites reported earlier). The formation of the above metabolites by *T. lanuginosus* suggested that this mould can be used as a model organism for drug metabolic study. Biotransformation of diclofenac was done by *Scytalidium thermophilum*; metabolites were confirmed by HPLC analysis of the extract of experimental flasks, and transformation in diclofenac was identified by LC-MS (ShanthiPriya et al. 2017). In view of the above, the thermophilic coprophilic fungus *S. thermophilum* is a potent fungus for biotransformation of diclofenac.

7.7 Biosynthesis of Nanoparticles

Due to cost-effectiveness and eco-friendly nature, biological formation of nanoparticles has currently gained immense interest. Nanoparticles are clusters of metal atoms, 1–100 nm in size usually, and highly promising in commercial products because of their broad-range applications. The properties of nanoparticles are based on their structure, composition, crystalline nature, shape and size. Pure metals in the form of nanoparticles are applied in various fields such as medicine – including antimicrobial agents, diagnostics, medical devices, treatment of several diseases and drug delivery – paints, textiles, biosensing, food processing, electronics and cosmetics. Metal origin nanoparticles are synthesized by biological, chemical and physical approaches, but chemical and physical methods are traditional methods for nanoparticle synthesis as compared to biological methods. Syed et al. (2013) reported biosynthesis of nanoparticles from the thermophilic fungus *Humicola* sp. by the reaction of fungus with silver ions which decrease the precursor solution, and as a result of this reaction, extracellular nanoparticles are formed. The interesting point in this investigation was that the researchers attained exclusive check on nanoparticle size ranging from 5 to 25 nm, so that when using them in biomedical applications, these nanoparticles in the glomerulus of kidneys will not obstruct and take a short time period to easily pass through the urine. These nanoparticles are harmless for normal and cancerous cells up to 50 μg/ml and will have varied uses in drug delivery and targeted drug delivery systems (Syed et al. 2013). Bio-inspired production of biomedically significant cerium oxide nanoparticles has been reported by Khan and Ahmad (2013) for the first time from thermophilic *Humicola* sp. on exposing the fungus to aqueous solution of oxide precursor cerium nitrate hexahydrate that results in the production of extracellular cerium oxide nanoparticles with Ce (IV) and (III) in mixed oxidation states as confirmed by XPS (X-ray photoemission spectroscopy).

The nanoparticles thus formed are naturally capped with protein release by fungus and that is why they are water dispersible and highly stable and do not agglomerate. The thermophilic *Humicola* sp. can be utilized for the synthesis of Gd203 nanoparticles at 50 °C (Khan et al. 2014). Along with fungal biomass, GdCl dissolved in water, and GdCl ionizes to Gd∗ and 3Cl. The fungus secretes oxidase enzymes in solution, and the mixture acts on Gd ions and results in the generation of Gd_2O_3 nanoparticles. These nanoparticles have irregular shape, displaying an overall quasi-spherical morphology, and they have also shown a role in site-specific drug delivery system for cancer therapy. This work of Gd_2O_3 biosynthesis was extended by Khan et al. (2014) to nanoparticles by bioconjugation of taxol. Gibson et al. (2007) and Hwu et al. (2009) have also reported the bioconjugation of taxol with gold and iron oxide nanoparticles. Taxol is the one of the most important anticancerous drugs used for lung, ovarian and breast cancer treatments, and the powerful anticancer effect of taxol is mainly due to its mode of action. This drug stabilizes microtubules by halting their depolymerization. Molnar et al. (2018) reported synthesis of gold nanoparticles by using either extracellular and intracellular fractions or the autolysate of 29 thermophilic fungi. The formation of nanoparticles of varied size (6–40 nm range); size distribution depends on the fungal strain and conditions of experiment, and they also found that *Thermoascus thermophilus* ATCC® 26413™ proved to be reliable in producing gold nanoparticles of uniform size and shape.

7.8 Antimicrobials and Other Bioactive Compounds of Biotechnological Interest

Svahn et al. (2012) reported that among 61 filamentous fungi, chiefly different *Aspergillus* spp. like *A. fumigatus* showed antimicrobial activity against vancomycin-resistant *Enterococcus faecalis* and *Candida albicans* and methicillin-resistant *Staphylococcus aureus* and extended-spectrum β-lactamase-producing *E. coli.* Guo et al. (2011) reported gliotoxin as a secondary metabolite from *Aspergillus fumigatus* recognized after molecular characterization and also the isolate 6-indole alkaloid from the thermophilic fungus *T. thermophilus* YM3–4. Compounds 1 and 2 were identified to be new analogues for the precursor notoamide E; compound 3 was a unique analogue of prechinulin; compound 4, for the very first time, was found as a naturally occurring cyclo (glycyl-tryptophyle), and for these talathermophilins, this fungus showed a special biosynthetic pathway. Two such types of alkaloids, talathermophilins A (1) and B (2), were reported from *T. thermophilus* YM1–3 and recognized by MS and NMR spectroscopic analysis (Chu et al. 2010). Guo et al. (2012) reported macrocyclic PKS-NRPS hybrid metabolites constituting a distinctive family of natural products, and these hybrid metabolites hold a 13-membered lactam supporting macrolactone, thermolides A-F (1–6). A large number of biomolecules with various industrial applications are produced by *M. thermophila.* In *M. thermophila,* osmotic adaptations were regulated by compatible solutes like amino acids and sugars. The analysis of fatty acids of

lipids from membranes contained comparatively a high percentage of unsaturated components, which are well known to be associated with high fluidity of membranes (Katapodis et al. 2003).

7.9 Future Perspectives and Conclusions

The thermophilic fungi are ubiquitous in nature and efficiently degrade organic materials of plants and have great potential in environmental management and biotechnology, although they are less explored organisms. Nevertheless, new genomic studies and programmes emphasize on enzymes of thermophilic fungi because of their applications in biofuel production and other commercial product formation which open up new approaches and direction for exploitation of these fungi. These fungi have a potential to secrete a variety of extracellular enzymes which make them efficient decomposers. Thermophilic fungi produce enzymes that are more efficient catalytically and are thermostable than their mesophilic counterparts. The availability of whole-genome sequences of thermophilic fungi provides an added advantage for genome mining. For attaining enhanced enzyme titres, synthetic biology and scale-up studies are used, and there is a possibility of applying bioinformatic tools for ameliorating the catalytic efficiency of the thermostable enzymes. Metagenomic approaches aid in unravelling their role and diversity in environments such as compost and soil. Detailed research investigations will be useful in recognizing the potential of these fungi in carrying out bioconversion of steroid and non-steroid compounds and also in unravelling intra- and inter-species differences and similarities in the metabolism of steroids.

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Deep Biosphere: Microbiome of the Deep Terrestrial Subsurface

8

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Abstract

Deep biosphere represents an unexplored realm of planetary life residing underneath the continental and oceanic crusts that constitutes majorly of prokaryotic life forms bacteria and archaea. Microbial communities which reside within various deep subsurface environments form a significant but largely unknown portion of the Earth's biosphere. While the shallow aquifer and sedimentary rock microbiome might get access to the nutrient pool available above ground, deep subterranean habitats hosted by crystalline rocks are severely constrained by the availability of photosynthetically derived nutrients. Deep subsurface microbiome underneath the continental crusts not only showed variations based on their geographic locations but also with respect to the abundance of various microbial populations and their metabolic properties. It is estimated that the deep biosphere microorganisms represent the largest pool of carbon, nitrogen, and phosphorous and constitute a critical component of biogeochemical engine of our planet. The aphotic deep dark microbial realm that has evolved possibly billions of years ago has developed unique metabolic repertoire for their survival. The deep biosphere microbiome is considered to be a portion of planetary life with extraordinary life-supporting system that works beyond our notion about biological and physical constraints. Advancement of techniques in microbial ecology has enabled us to decipher deep subsurface microbiome which resides up to several kilometers below the surface using both cultivation-dependent and cultivation-independent techniques. In this chapter, we have summarized our understanding of the deep biosphere microbiome within terrestrial subsurface. Habitability of life within the

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deep subsurface has been discussed considering the major metabolic routes deployed by the microorganisms. Cultivation-dependent and cultivation-independent studies and their requirement and outcome from various exploratory researches have been documented. Techniques used for sampling the subsurface microbiome are discussed, highlighting the role of possible contamination during drilling and subsequent postcore extraction processes. Lastly, applications of deep subsurface microbiome research in achieving better sustainability and biotechnological innovations are discussed.

Keywords

Deep biosphere · Metabolic processes · Metagenomics · Enrichment · Drilling process-contamination · CO_2 sequestration · Waste repository · Bioprospecting

8.1 Introduction

Variations and complexities of life on Earth have always surprised biologists. Even the intricacies of the simplest known organism are so pronounced that it leaves us astounded. Microbes are the earliest known life forms, which is evident from the fossil records. Microbes consist of both prokaryotes and eukaryotes, of which prokaryotes are thought to be the first living organisms on our planet. It is estimated that the number of prokaryotic cells residing on our planet ($4–6 \times 10^{30}$) (Whitman et al. 1998) is much higher than the number of planets present in our galaxy (1×10^{11}) (Cassan et al. 2012). Projections show that the total population of prokaryotes harbors $350–550 \times 10^{15}$ g carbon (accounting for 60–100% of the estimated total carbon in plants), $85–130 \times 10^{15}$ g nitrogen, and $9–14 \times 10^{15}$ g phosphorous (accounting for tenfold more nitrogen and phosphorous than plants) making them the largest pool of these nutrients in the living organisms (Whitman et al. 1998). Microbial assemblages can vary and diverge from place to place and create distinct biogeographic patterns (Green et al. 2008). Based on various evolutionary circumstances, biogeographic patterns are hypothesized to expand or regress owing to the effects of ecological and evolutionary forces at the genomic level (Ramette and Tiedje 2007). Traditional opinion on microbial biogeography has been that “Everything is everywhere, but the environment selects” (Baas-Becking 1934). However, it is debatable whether distribution of microbial populations over space results from environmental selection or if dispersal of microorganisms is restricted and affected by geographical barriers and other incidents in the geologic past (Eisenlord et al. 2012). Events in the geologic past may give rise to niche-specific diversity pattern through isolation and genetic divergence. Microbial diversity pattern often varies owing to uneven and unequal distributions of microbes. Restrictions in even and equal distribution of microbes suggest that factors shaping microbial community structure are more complex than the adaptive evolution through natural selection (Eisenlord et al. 2012).

Microbes are an important support for the Earth to function and microbial diversity is an unseen resource that deserves greater attention (Mishra 2015). Study on microbial diversity will not only help to maintain and conserve global genetic resources but also

will help us to know the unknown (Colwell 1997). Microbes reside in different spheres of our planet of which the major proportion of it inhabits the subsurface environment (Whitman et al. 1998). Permanent darkness persists in the subsurface provinces which are separated from the light-driven surface world (Edwards et al. 2012). The ecosystems which sustain in the subsurface environment are often referred to as deep biosphere. Hoehler and Jørgensen in 2013 described deep biosphere as "the set of ecosystems and their organisms living beneath the upper few meters of the solid earth surface." Extent of life in subsurface is much deeper than it was presumed earlier. It was thought that life is a surface phenomenon and sustenance of life even by the "hardy prokaryotic types" is not beyond tens of meters below the surface (Jannasch et al. 1971). In the 1990s and early 2000s, it became much more evident that life in the deep biosphere is ubiquitous and comprises a metabolically and genetically diverse microbial community (Parkes et al. 1994; Takai et al. 2001; Fry et al. 2008; Reith 2011). Nevertheless, the facts about lower depth limit of deep biosphere, energy sources sustaining microbial communities, and the link between microbial diversity/function and geochemical/geological factors remain elusive (Reith 2011). However, the knowledge that we have is that the deep subsurface is characterized by extreme conditions where the microorganisms have developed various mechanisms to deal with different physical and chemical constraints such as high pressure, high temperature, limited energy and nutrient availability, extreme acidity and alkalinity, metal toxicity, and radioactivity (Pikuta et al. 2007).

8.2 Habitability of Life in Deep Subsurface

Life in deep biosphere is often exposed to different extremes. Deep biosphere environments are generally characterized by aphotic and oligotrophic nature frequently having elevated temperatures and pressures. Other extremities include water scarcity, radiations, high salinity, and presence of degenerative substances which might be the limiting factors for sustenance of life in deep subsurface. It is thought that microorganisms that reside in such extremes must have evolved mechanisms of adaptation that makes themselves suitable to thrive under such harsh conditions (Kieft 2016). Interestingly, many of the inhabitants of these extreme environments can not only tolerate these harsh conditions but also often require those conditions for their survival (Rampelotto 2013). Knowing the extremities in the subsurface, it has also been postulated that microbial cells in deep biosphere might enter into a stage of "semi-senescence" due to severe nutrient deprivation which might extend their doubling time in the range of hundreds to thousands (Chivian et al. 2008).

Nutrient availability in the deep biosphere regime is limited and restricted. Microbes residing in such extreme habitats typically occupy the fractures or pore spaces with nutrients made available either from the rock/sediments and/or through transportation (via available interconnections) in the form of dissolved gases, solutes, or colloids (Fredrickson and Balkwill 2006). But often it has been found that sources of nutrients in the deep biosphere vary in different locations. Main sources of nutrients in deep provinces are either biogenic or geogenic in nature. Possible geological and biological processes that support the sustenance of life in the subsurface provinces mainly with

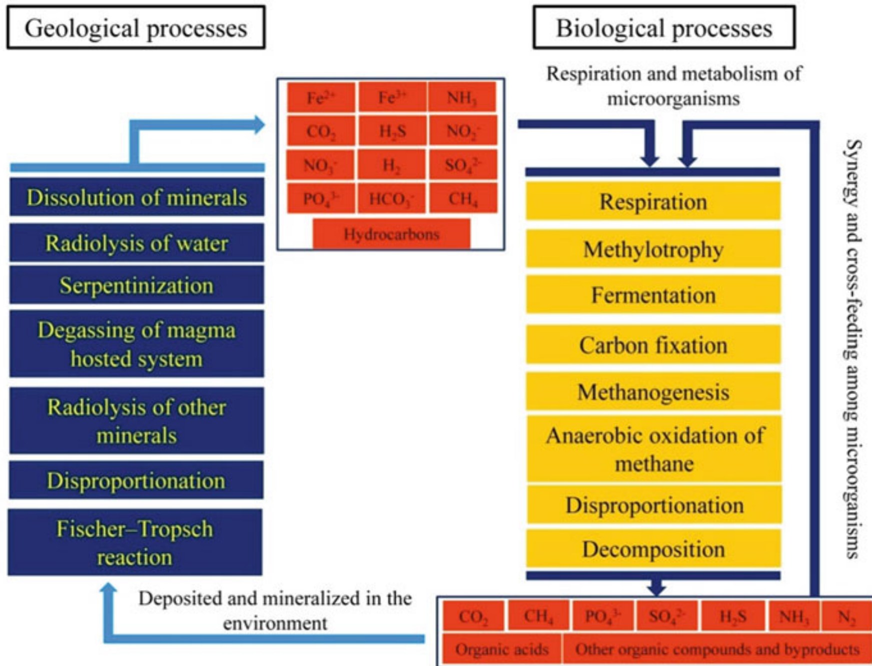

Fig. 8.1 Possible geological and biological processes that support the sustenance of life in the subsurface igneous provinces

respect to igneous provinces are mentioned in Fig. 8.1. Deeply buried organic matter can serve as a nutrient source for the subsurface life, but its presence in the subsurface environment is rare. Geologic events create an opportunity for introduction of nutrient in such oligotrophic environment. Seismicity and other processes like continental drifts create faults, fractures, and fissures which allow water to percolate in the deep subsurface. The infiltrated water from the surface not only provides the basis of life in the subsurface but also carries nutrients for microbial cells to thrive. Abiotic processes like serpentinization, radiolysis of water, oxidation of minerals, mineral dissolution, and degassing of magma-hosted systems help in the formation of different gases like H_2, CO_2, CH_4, and H_2S. These gases can be utilized by a specific group of microorganisms for their survival in the deep biosphere. Byproducts of these microbial groups are utilized by other populations for their sustenance. These kinds of ecosystems are mainly fuelled by hydrogen (which might be geogenic or biogenic in nature) and are termed as hydrogen-driven subsurface lithoautotrophic microbial ecosystems (SLiMEs) (Stevens and McKinley 2000; Nealson et al. 2005). The main stakeholders of these ecosystems are sulfate reducers, methanogens, and anaerobic methane-oxidizers. Sulfate reducers involved in these systems are obligate or facultative anaerobes which use the mechanism of dissimilatory sulfate reduction (DSR). DSR is a form of anaerobic respiration where sulfate is converted to sulfide. Microorganisms harboring sulfate adenylyltransferase (*sat*), adenylyl-sulfate reductase (*apr*), and dissimilatory sulfite reductase (*dsr*) genes are generally involved in this

process. DSR is mainly observed in bacteria affiliated to *Deltaproteobacteria* (genera *Desulfovibrio*, *Desulfomonile*, *Desulfopila,* and others) and *Firmicutes* (*Desulfotomaculum*, *Desulfosporosinus*, *Desulforudis*, and others). In hydrogen-driven ecosystems, often such reducers are fuelled by geogenic hydrogen or hydrogen liberated by anaerobic methane oxidizers. The process of anaerobic methane oxidation (AOM) is restricted to the domain archaea, and most of the members are closely related to class *Methanomicrobia* (ANME-1, ANME-2, and ANME-3). AOM was also reported in other archaeal members such as *Candidatus* Methylomirabilis oxyfera, *Candidatus* Methanoperedens nitroreducens, and Marine Benthic Group D (Cui et al. 2015). All known ANME members harbor methyl-coenzyme M reductase (*mcr*) gene which is the key gene for methanogenesis, and it is postulated that *mcr* present in these archaea is responsible for anaerobic methane oxidation by a process called reverse methanogenesis (Cui et al. 2015; Timmers et al. 2017). Though AOM was first found to be coupled with sulfate reduction, later studies reported coupling of AOM with denitrification and metal ion (Mn^{4+} and Fe^{3+}) reduction (Cui et al. 2015). Anaerobic methane oxidizers in the deep subterranean environment are driven by biogenic or abiogenic methane. Biogenic methane is liberated by methanogens which are one of the prominent residents of deep biosphere. Methanogenesis is restricted to the domain archaea and mainly affiliated to seven orders (*Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanocellales*, *Methanopyrales*, and *Methanomassiliicoccales*) of phylum *Euryarchaeota*. Later studies revealed that methanogenesis is phylogenetically widespread and also found in phylum *Bathyarchaeota* (formerly Miscellaneous Crenarchaeota Group), *Verstratearchaeote*, and other Candidatus groups (Vanwonterghem et al. 2016). Central role of methane metabolism is played by methyl-coenzyme M reductase complex. Substrates for methanogenesis mainly include H_2/CO_2 (hydrogenotrophic), acetate (acetoclastic), and methylated compounds (methylotrophic). In subsurface oligotrophic ecosystems, it is frequently seen that these substrates are produced by fermentative group of microorganisms. Fermentation is an anaerobic process where sugar is consumed by an organism to produce CO_2, H_2, organic acids, alcohol, or combination of either. This process is not only widespread across different taxonomic groups of bacterial domain but also found in single-celled eukaryotes such as yeast. Some of the known organisms which are prominent fermentors are *Saccharomyces* (ethanol fermentation), *Lactococcus* (homolactic acid fermentation), *Leuconostoc* (heterolactic acid fermentation), *Propionibacterium* (propionic acid fermentation), *Escherichia* (mixed acid fermentation), *Enterobacter* (2,3-butanediol fermentation), *Clostridium* (butyrate fermentation and acetone-butanol fermentation), and *Acetobacterium* (homoacetic acid fermentation). Some of the genes which play key roles in fermentation are lactate dehydrogenase (*ldh*), pyruvate dehydrogenase (*pfl*), alcohol dehydrogenase (*adh*), acetate kinase (*ack*), phosphoenolpyruvate carboxylase (*ppc*), and malate dehydrogenase (*mdh*). In addition to these pathways, denitrification and ammonification (for respiration and assimilation) are frequently observed in subterranean deep biosphere. Both these processes not only play an important role in nitrogen cycle but also are commonly coupled with processes of other subterranean biogeochemical cycles. Some of the common denitrifiers are affiliated to *Pseudomonas*, *Micrococcus*, *Achromobacter*, *Serratia*, and *Thiobacillus*. Major genes involved in denitrification

processes are nitrate reductase (*nar*), periplasmic nitrate reductase (*nap*), nitric oxide reductase (*nor*), nitrous oxide reductase (*nos*), and nitrite reductase (*nir*). Dissimilatory nitrate reduction to ammonium (DNRA) is an important ammonification process in the deep which is generally found in anoxic environment and observed in both prokaryotes (*Beggiatoa*, *Thioploca*, *Candidatus Nitrosocaldus yellowstonii*, and others) and eukaryotes (*Aspergillus terreus*, *Fusarium oxysporum*, *Cylindrocarpon tonkinense*, and others). Common marker gene used to detect bacterial DNRA is nitrite reductase (cytochrome c-552) (*nrf*A) which is reported from different subterranean deep biosphere sites (Momper et al. 2017; Lau et al. 2016).

Different physicochemical conditions in the subsurface environment suggest that chemolithoautotrophic microorganisms are the main dwellers in the subsurface provinces. S^{2-}, NO_2^-, NH_3, Fe^{2+}, and H_2 are widely available reduced inorganic compounds in the subsurface environment which can act as an energy source for the chemolithoautotrophs. Reducing inorganic compounds may be geogenic or biogenic in nature. Geogenic sources of these reducing compounds are mainly from mineral (like pyrite, phyllosilicates, etc.) dissolution, water-rock interaction, and radiolysis, whereas the biogenic sources are the products of sulfate-reducing, denitrifying, nitrogen-fixing, iron-reducing, and fermentative bacteria (Nealson et al. 2005; Chivian et al. 2008; Lau et al. 2016). Chemolithotrophs can be either obligate or facultative in nature which are phylogenetically diverse and play an important role in different biogeochemical cycles in subsurface provinces. Some of the established chemolithotrophs are *Nitrospira*, *Nitrobacter*, and *Nitrosomonas* (ammonia oxidizers); *Gallionella*, *Thiobacillus ferrooxidans*, and *Leptospirillum ferrooxidans* (iron oxidizers); *Hydrogenobacter thermophilus*, *Aquifex aeolicus*, and *Hydrogenovibrio marinus* (hydrogen oxidizers); and *Acidithiobacillus*, *Thiomonas*, and *Thiobacillus* (sulfur oxidizers). Common genes involved in chemolithotrophy are ammonia monooxygenase (*amo*), sulfur-oxidizing protein (*sox*), sulfide:quinone oxidoreductase (*sqr*), hydrogenase expression/formation protein (*hyp*), [NiFe] hydrogenase, and [FeFe] hydrogenase. These chemolithoautotrophs often derive cellular carbon from carbon dioxide. Some of these organisms also harbor genes to fix bicarbonate. There are several CO_2 fixation pathways which are observed in aphotic subterranean provinces. One of the earliest known pathways for CO_2 fixation by microorganisms is Calvin-Bassham-Benson (CBB) cycle which uses 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to assimilate CO_2 (Bassham and Calvin 1957). Other CO_2 fixation pathways frequently reported from deep biospheres are reductive tricarboxylic acid (rTCA) cycle, Wood-Ljungdahl (WL) pathway, and 3-hydroxypropionate (3-HP) cycle (Momper et al. 2017; Lau et al. 2016; Purkamo et al. 2015). In addition to CO_2 fixation, the 3-HP pathway exclusively fixes HCO_3^-. Some of the key marker genes used to detect these pathways are ribulose-bisphosphate carboxylase (*rbc*) (CBB cycle), ATP citrate (pro-S)-lyase (ACLY) (rTCA cycle), acetyl-CoA synthase (*acs*) (WL pathway), and acetyl-CoA carboxylase (*acc*) (3-HP) cycle. Geogenic sources of carbon dioxide and bicarbonates in the subsurface provinces are mainly from degassing of magma-hosted system and dissolution of calcite minerals, respectively. Biogenic contributors of CO_2 in the subsurface ecosystems are mainly the fermentative and anaerobic methane-oxidizing bacteria. Chemolithoautotrophs can be both aerobic and anaerobic in nature. Though oxygen is limited in the subsurface provinces, presence of terminal electron

acceptors (TEA) like NO_3^-, NO_2^-, Fe^{3+}, and SO_4^{2-} allows facultative anaerobes and obligate anaerobes to thrive in the subsurface depending on the availability of TEA.

Deep biosphere is often deprived of organic carbon. Dearth of organic carbon in the subsurface environment gives us a sense that microbial life in deep biosphere has prevalent chemolithoautotrophic lifestyle, but this is not always true. Heterotrophic microorganisms have also been reported from subsurface environment (Hallbeck and Pedersen 2008; Nyyssönen et al. 2014; Purkamo et al. 2015). Metabolic intermediates or products of chemolithoautotrophic metabolism can fuel the heterotrophs in the subsurface. Heterotrophic microbial groups can also be fuelled by geogenic hydrocarbons generated by Fischer–Tropsch reaction where liquid hydrocarbons are created from carbon monoxide and hydrogen (Purkamo et al. 2016).

Considering the extremities, it is often thought that specialist groups of organisms having less diverse populations are known to reside in the subsurface environment. Though single-species ecosystem has been reported from South African Gold Mine (Chivian et al. 2008), most of the reports suggest that organisms in nutrient-deprived stressed subsurface ecosystems prefer to work in synergy. Often co-occurrence of different microbial populations in the subsurface environment is observed which substantiates the fact of cross-feeding and mutualistic behavior in the subsurface. Co-occurrence of microbial populations might also be attributed to the nutrient availability and environmental amicability where different species exploit the same resource and prefer to reside in a similar environment.

8.3 Cultivation-Dependent Studies

8.3.1 Cultivable vs Uncultivable

Investigation on deep biosphere microbiome remained more cultivation centric for a considerable period (Jannasch et al. 1971; Cragg et al. 1990; Parkes et al. 1994; D'Hondt et al. 2004; Hallbeck and Pedersen 2008). In the early 1990s, the first report on changes in microbial activity with depth, biogeochemistry, and estimation of cultured biodiversity was published (Cragg et al. 1990; Parkes et al. 1994) showing that there exists a clear link between biological activity and the availability of organic carbon and terminal electron acceptors in the deep subsurface biosphere. D'Hondt et al. (2004) reported the diversity of microbial communities and numerous energy-yielding activities that occur in deeply buried sediments of oceanic environment. Using the samples recovered from one of most representative sites for Earth's ocean (Ocean Drilling Program (ODP) Leg 201: equatorial Pacific Ocean and the continental margin of Peru) wherein the water depths range from 150 m to 5300 m, elaborate analysis of microbial activities including metabolic requirements has been reported. Sediment samples were obtained from sub-seafloor depth from 0 to 420 m, temperature from 1 to 25 °C, and age from 0 to 35 million years ago (Ma). Presence of prokaryotic cells occurring throughout the sampled sediment column was noted in every location. With culture-based methods, these investigators have shown that rates of activities, cell concentrations, and populations of cultured bacteria may vary consistently from one sub-seafloor environment to another. A major role of

photosynthetically derived substances from surface in providing necessary electron acceptors and electron donors for microbial metabolism was noted. Hallbeck and Pedersen (2008) reported that microorganisms should be considered as an insepara-ble part of the "hydrogeochemical modeling." They have developed and tested several culture-dependent methods to estimate the total number of microbial groups, to quantify their biomass amount in groundwater, to study their diversity, and to find out the type of metabolic profile they belong to. Recently, another interesting study with Fennoscandian shield deep subsurface groundwater samples has hypothesized that microbial communities residing in deep subsurface Fennoscandian shield are distinctive to each site or area (Purkamo et al. 2018). The role of iron-oxidizing bacterial communities and methanogenic and ammonia-oxidizing archaeal groups was identified. The role of geochemistry in shaping microbial communities and their functions were highlighted.

Isolation and characterization of microbial populations using various enrichment or direct isolation-based methods have enriched our understanding of this section of microbial world. We have looked into the 16S rRNA gene inventory within the Ribosomal Database Project Database (Fig. 8.2). With a search keyword of "Deep Biosphere," 269 sequences so far retrieved from various isolates were found. These organisms are taxonomically affiliated to six phyla with the maximum hits belonging to the phylum *Thermotogae*, followed by proteobacterial members.

The following section describes a brief outline of the cultivation-based microbiome study and importance of getting the appropriate medium for growth and cultivation of deep biosphere organisms. In natural habitats, there exist microorganisms that can be differentiated into distinct categories based on their culturability (Madsen 2008; Stewart 2012). The small fractions of the total microorganisms that readily form colonies on agar plates are the ones that grow and are known as the cultured organisms. Microbial growth requires proper resources, especially a carbon source, nutrients, electron donors, and electron acceptors, and necessary interactions among the organisms and their abiotic environment. Cultured microorganisms are those that have been successfully isolated and purified in the laboratory. The remaining ones that do not grow on readily formulated medium are known as uncultured microorganisms. Uncultured microorganisms are the ones for which no appropriate growth medium has been

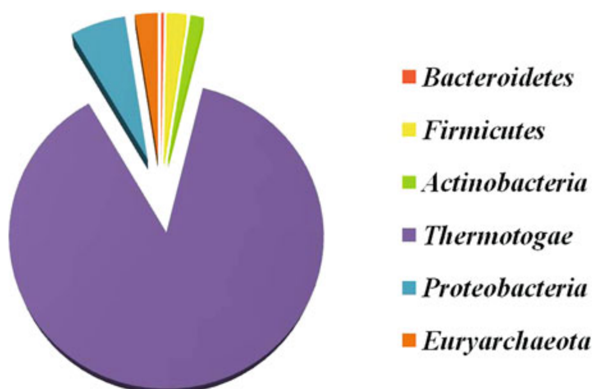

Fig. 8.2 Taxonomic affiliation of microorganisms isolated from deep biosphere

devised (Stewart 2012; Vartoukian et al. 2010). The uncultured category can be further divided into culturable and nonculturable. Culturable microorganisms are the ones that can be cultured when an optimized growth medium, which matches the organism's nutritional needs, is used for cultivation. Key physical and chemical growth conditions must also be provided. Nonculturable organisms are the ones whose physiological state prevents them from being cultured, i.e., they do not grow even when proper growth conditions are provided. Nonculturable cells are also known as dormant (Madsen 2008).

Figure 8.3 illustrates the approaches for isolation of different categories of microorganisms from deep subsurface rock samples based on their cultivability.

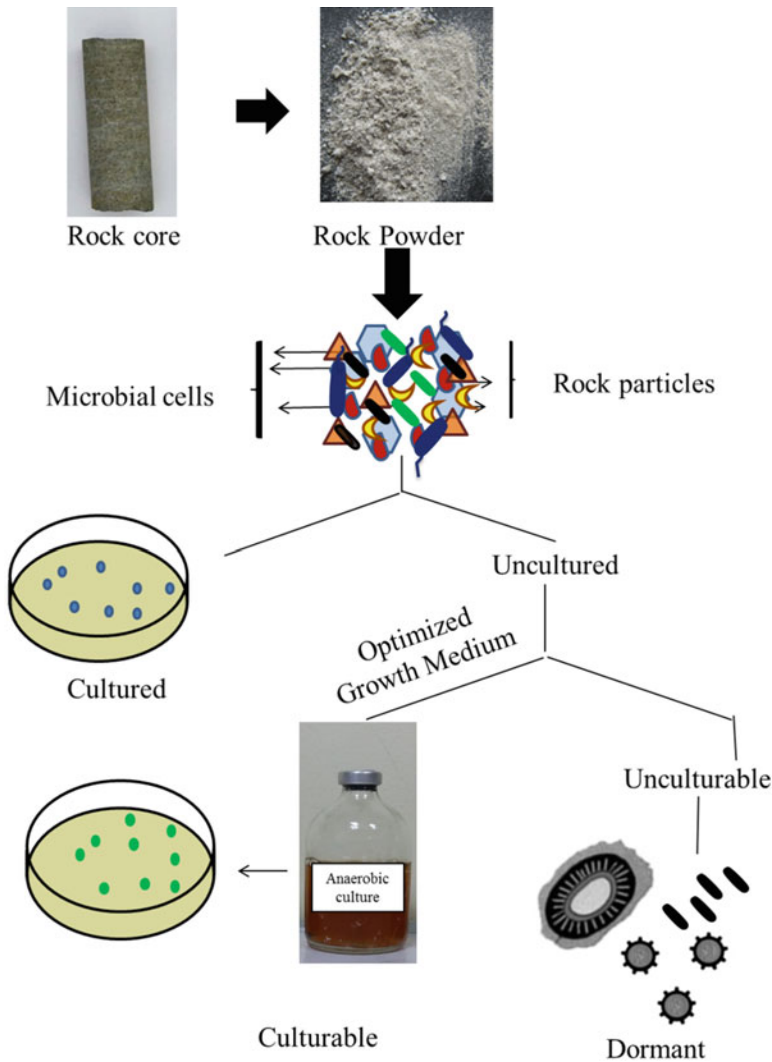

Fig. 8.3 Different categories of microorganisms (based on their cultivability) which could be studied

8.3.2 Requirements for Cultivation and Growth of Microorganisms from Deep Subsurface

For growth to happen, microbes need energy and other essential material resources which in turn can be obtained from raw materials and nutrients. A culture medium is one such preparation which provides the organism with necessary requirements of nutrients. Specialized media preparations are essential in the isolation, identification, and characterization of microorganisms from deep oligotrophic subsurface samples. Although all microorganisms need sources of energy, carbon, nitrogen, phosphorus, sulfur, and various other major and trace elements, the exact composition of a proper medium depends on the type of organism to be cultivated, as nutritional requirements of different group of organisms vary greatly. Idea about the microorganism's normal habitat can often be useful in selecting an appropriate culture medium because the nutrient requirements are very much linked to its natural surroundings (Prescott et al. 2002).

Exploring the microbial diversity in nature and finding the ecological connections between environmental geochemistry and microbial communities will help us to unveil how organisms survive and thrive in natural habitats and provide insights into the development and expansion of life on Earth. For many years, attempts have been made by researchers to use molecular techniques to find the relationships between microbial diversity and physicochemical and geochemical parameters, indicating that environmental variables such as moisture content, conductivity, pH, temperature, and concentrations of electron acceptors and donors can have strong influences on the phylogenetic differentiation among microbial cohorts in natural habitats. These studies have provided insights into the factors, both physical and chemical, that dictate what kinds of metabolisms are possible in a natural setup which in turn determines the pathways of energy harness by the inhabiting microbes. It also helped in understanding the response microbes have toward temporal shifts in the environment geochemistry. In fact, even in "extreme" environments, the distribution of microbial populations and communities is shaped by the prevailing environmental conditions (Richards et al. 2014; Stevens et al. 1993).

Conversely, microbes shape their geochemical surroundings through their metabolic interactions and growth needs, controlling every facet of redox, metal, organic, nutrient, and trace element components, which determines the geochemical and mineralogical composition of the surroundings. Microbial evolution has occurred in concert with changing geosphere conditions—microbes have been the major drivers causing shifts in the chemistry of oceans, continents, and atmosphere (Knoll 2003a, b). The role of microbes is critical for element cycling in any environmental system. A combination of different experimental approaches to interrogate microbial activity (through physiology, genetics, culturing, and microscopy) and geochemistry (aqueous, mineral, isotope geochemistry) has been developed to address these critical and significant interactions between microbes and their surroundings. Changes in the environment occur when interactions between physical

entities exceed its buffering capacity. Environmental change, in turn, feeds back on biology, creating shifts in microbiological communities. In nature, energy and nutrient flow is intricately coupled to complex geochemical reactions and processes (mineral precipitation and dissolution reactions, absorption reactions, redox reactions, etc.) that can affect the microbial growth (Istok et al. 2010). In turn, microorganisms also influence the chemical and physical properties of their surrounding environment (Ham et al. 2017). Microorganisms, residing in minute fractures in the deep crystalline crust, gain energy by following diverse metabolic processes (Kieft 2016). Microorganisms can interact with the environment acquiring different nutrients, electron donors, and electron acceptors such as molecular oxygen, nitrate, metal oxides, sulfate, sulfur, carbon dioxide, or water. Metabolic and growth interactions are not the only interaction happening in the environment. Microbes interact within themselves following different mutualistic relations, which help each of the interacting groups to survive in a particular environment and thrive in nature. Sharing of electron donors and acceptors, interspecies hydrogen ion transfer, and utilization of metabolic byproducts are some of the interactions which play a major role in the formation of a microbiome inside the deep terrestrial subsurface. Life in the deep subsurface is partially dependent on the supply of carbon and energy from the surface even though there are evidences that microbial life habituating deep in the crystalline rocks can derive its energy from autotrophic processes independent of photosynthesis and can also utilize hydrogen as an energy source (Stevens 1997; Pedersen 1999). Figure 8.4 illustrates some interactions which go on in between environment and organism and within different microorganisms residing inside the Earth's crust.

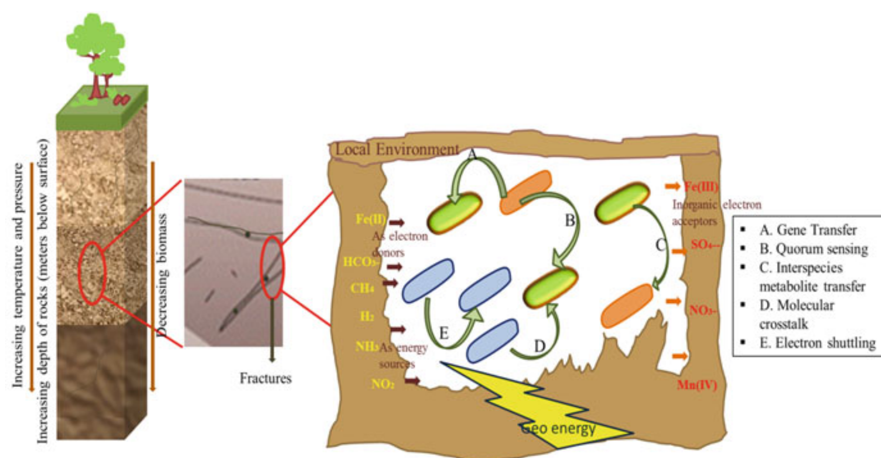

Fig. 8.4 Processes and interaction (inter cellular interaction and interaction with the local environment) that occur within the deep subsurface which fuels the biosphere

8.3.3 "Omics"-Based Technologies: A Helping Hand Toward Understanding the Organisms, Their Metabolisms, and Their Growth Requirements

In order to achieve higher levels of cultivability, necessary clues may be obtained from cultivation-independent, omics-based microbial community studies. High-throughput next-generation sequencing technologies have rapidly become a substantial tool for studying diversity and distribution of microbial ecosystems in the environment. Large-scale sampling and deep sequencing of microbial communities from different geographic regions and areas have revealed that there are specific effects of geochemical factors on the microbial diversity patterns and community composition in the environment (Liu et al. 2014; Joseph et al. 2003; Vartoukian et al. 2010). These technologies have enabled the generation of large amounts of genetic information on microorganisms without the need to grow cultures in the lab. Armed with these technologies, one can generate draft metabolic network for organisms directly from genome annotations and shed light on the procedures to enhance growth of cultivable microbes. A closer look into the 16S rRNA gene inventory within the Ribosomal Database Project Database, a search string of "Deep Biosphere" retrieved 1050 matches of uncultured microorganisms belonging to various deep subsurface regions. These organisms are taxonomically affiliated to various phyla with the maximum hits belonging to the archaeal phylum Crenarchaeota, followed by other archaeal phyla. As for the members of the bacterial domain, the maximum members belonged to the phylum *Microgenomates* (Fig. 8.5).

This kind of "omics"-based approach can help us in knowing the organisms residing in a particular habitat and hence help in formulating specific growth medium to

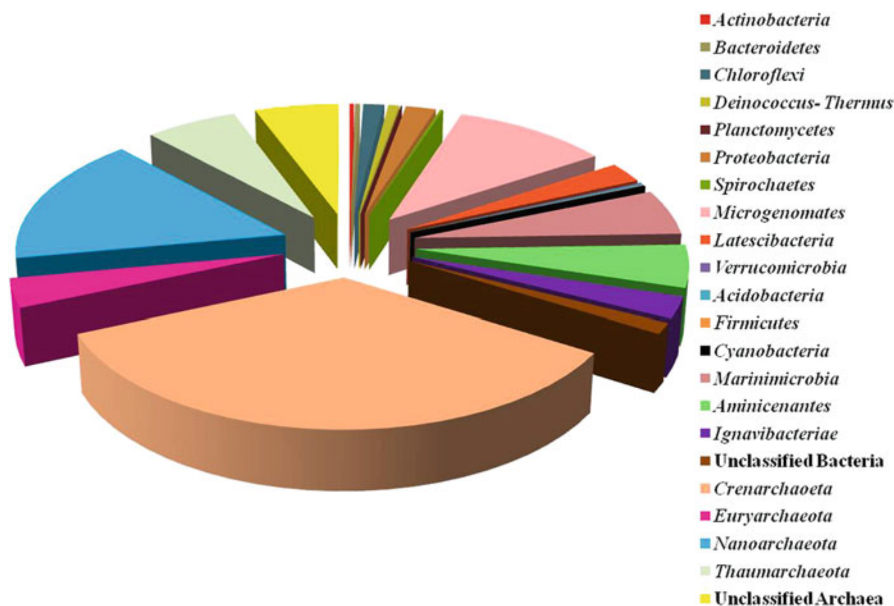

Fig. 8.5 Taxonomic affiliation of uncultured microorganisms from deep biosphere

cultivate the uncultured population. Isolation of pure microbial cultures and cultivating them in the laboratory on defined medium is used to characterize the metabolism and physiology of organisms fully. But, as it is said that it is "easier said than done," identifying an appropriate growth medium for a novel isolate remains a challenging task. Even organisms with sequenced and annotated genomes can be difficult to grow, despite the ability to build genome-scale metabolic networks that connect genomic data with metabolic function. The term the "great plate count anomaly" was coined by (Staley and Konopka 1985) to describe the difference in magnitude between the number of cells from environmental samples that form colonies on agar media and the numbers countable under the microscope. One of the most significant explanations for the "great plate count anomaly" is that many of the microbial species that can grow in natural settings are not adapted for growth in media containing high concentrations of complex organic carbon, i.e., the medium components are inadequate. It can also be such that the species that would otherwise be "culturable" may fail to grow because of the inability to adjust to the conditions found in the medium used for the plate counts. These microorganisms may need oligotrophic or other fastidious conditions to be successfully cultured. These microbial strains maybe are common in nature but can only be cultivated by specialized techniques (Spiegelman et al. 2005). An important requirement of culture-dependent study is the growth medium. For proper growth of most of the organisms present in an environmental sample, the media for growth should be similar in nature to the surrounding habitat. The medium should more or less mimic the physiological, chemical, and environmental conditions of the ecosystem, for example, geochemistry of the rock samples and environmental conditions of the site should be studied. The hydrologic and geologic properties of the samples should be adequately understood to predict the distribution and physiologies of the microorganisms throughout the depth and also the mechanisms involved in their colonization (Colwell et al. 1997).

8.3.4 Medium Formulation Based on Extensive Study of Local Geochemistry

The repertoire of prokaryotic life found in the subsurface and sub-seafloor biosphere by cultivation-independent molecular methods is much greater than obtained by standard laboratory culture methods (enrichment setups and isolation procedures). Also, populations obtained so far using cultivation-based methods represent only a very small subset of those revealed by molecular methodologies and culture-independent studies (D'Hondt et al. 2004). Yet methods for analyzing microbial metabolic processes and their outcome are being developed, tried, and tested in situ conditions (Hallbeck and Pedersen 2008). Medium formulations, enrichment culturing, and different other isolation procedures are being used from the early days of this deep subsurface research to peek into this world of unknown habitants and study them. According to Stevens et al. (1993), geochemical processes which may be interdependently controlled with microbiological processes can contribute toward formation of a specific condition of the sampling site. To stay alive, grow, and propagate, microorganisms transform several components present in their local environment, between different reduced and oxidized

states. Microbiological growth and enhancement depend on the energy sources and electron acceptors present (Madigan et al. 2006). Organic carbon (including methane) and reduced inorganic molecules (including H_2) are possible energy sources in the subterranean environment (Hallbeck and Pedersen 2008). Table 8.1 highlights different examples of case studies where formulation of medium was done after extensive study of local geochemistry of the deep subsurface regions.

8.3.5 Nature of Organisms Recovered from Diverse Deep Terrestrial Subsurface Environments Through Enrichment Studies

Study of the environment deep beneath the Earth's surface may provide an opportunity in understanding the mechanism which helps organisms to survive in extreme and apparently nonfavorable conditions. There is a lot of evidence which supports the presence of life which is ubiquitously distributed deep inside the Earth's crust. It has also been suggested that this life is dependent on lithogenically and geogenically produced energy compounds to sustain their existence (Colman et al. 2017). The rock minerals play a critical role in providing the different growth elements which in turn helps in sustenance of life in this extreme habitat. This biosphere consists of a diverse group of organisms which mostly follow the anaerobic mode of respiration. Depending on the type of mineral which predominates, organisms like sulfate reducers, iron reducers, nitrate reducers, and acetate producers can be found. Presence of acetoclastic and hydrogenotrophic methanogens can also be seen in this biosphere.

Study of microbial community in rock-hosted deep terrestrial subsurface environment is limited. Among the published literature that has discussed about deep subsurface biosphere, some of the works were selected. Major deep biosphere culture dependent studies undertaken in Asia took into consideration parts of Japan and China. Chinese Continental Scientific Drilling Project at China is one of the deepest (2026 m) and earliest explored subsurface site in Asia (Zhang et al. 2005). Subsurface environment of this site was mainly dominated by proteobacterial members. The presence of *Bacteroidetes* and *Planctomycetes* was also observed. Iron-reducing bacteria were observed which thrived in thermophilic and alkaliphilic conditions. As per reports by Fukuda et al. (2010), several studies were conducted in mine environments and established Underground Research Laboratories (URL) in Japan. They suggested the presence of members of *Proteobacteria* and *Firmicutes* which could survive in alkaliphilic conditions. Piceance Basin, western Colorado, USA, North America, was explored to search for microbial communities where presence of anaerobic thermophilic sulfate-reducing bacteria was reported (Colwell et al. 1997). Similar studies were reported in basaltic aquifers of Snake River Plains (Lehman et al. 2004). These studies revealed presence of bacterial members which included heterotrophs, hydrogen oxidizers, iron reducers, etc. Subsurface sedimentary rocks of Antrim Shale harbored methanogenic communities (Waldron et al. 2007). On exploration of deep mine environment of North America, like

Table 8.1 Case studies dealing with formulation of proper growth medium based on geochemistry of samples

Local geochemistry	Media formulated	Organisms cultivated successfully/types	References
Alkaline pH, presence of sulfate, iron TIC>TOC, temperature around 29 °C	Artificially formulated. Containing five different electron acceptors (O_2, Fe(III), NO_3^-, SO_4^{2-}, HCO_3^-) and four groups of Electron donors (fermentation products, monomers, polymers, aromatics) in a mineral salts medium at pH 9.5 Incubation at 30 °C	Sulfate reducing bacteria (SRB)/Nitrate reducing bacteria (NRB)/Iron reducing bacteria (IRB)/ bacterial populations utilizing varied carbon substrates	Stevens et al. (1993)
Near-neutral pH, has presence of sulfate, manganese, iron, hydrogen, high TOC	Medium formulated which mimicked the in situ groundwater chemistry for optimal microbial cultivation Medium for NRB, SRB, IRB, Manganese reducing bacteria (MRB), Autotrophic acetogens (AA), Autotrophic methanogens (AM), Heterotrophic acetogens (HA), Heterotrophic methanogens (HM), etc., pH around 7	SRBs/NRBs/IRBs/ MRBs/acetogens and methanogens	Hallbeck and Pedersen (2008)
Contains sulfide, sulfate, nitrate, iron, methane, H_2/CO_2	MG1 medium was supplemented with 20 mM acetate and a headspace composed of N_2:CO_2, medium MG2 with the gas mixture H_2: CO_2 and medium MG3 was supplemented with a solution composed of propionate: Butyrate: Methanol and headspace composed of N_2:CO_2. The basal medium was same for all. Incubation of cultures was done at 30 °C	*Proteobacteria*, *Actinobacteria*, *Rhizobium*, *Nocardioides*, *Desulfovibrio*, *Brevundimonas*, etc.	Leandro et al. (2018)
Presence of sulfate, CH_4, acetate, H_2/CO_2 Temp: 29 °C in	MJS medium for cultivation of methanogens with methanogenic substrates	ANME–I organisms	Ijiri et al. (2018)

(continued)

Table 8.1 (continued)

Local geochemistry	Media formulated	Organisms cultivated successfully/types	References
sediments and around 60 °C in porewater	like H_2/CO_2, acetate, formate, etc., pH around 7 and temperature 25 °C/ 55 °C		
Alkaline pH, temp 10–20 degrees, low organic carbon content, sulfate conc. Increased with depth. NH_4^+ ions were present in detectable amounts	Artificial medium formulated for growth and enrichment of aerobes, obligate and facultative anaerobes, pH 7.5. Incubation was done at 20 °C	Obligate anaerobes, SRBs, etc.	Pedersen and Ekendahl (1990)

Lupin Au mine (Canada), Onstott et al. (2009) reported the presence of organisms which can reduce sulfate as a part of their metabolism or can tolerate high salt concentration. Fennoscandian shield which is present in the northern part is the most studied location in the continent of Europe. Many investigations related to deep biosphere have been done in this location that has increased our understanding in the field of deep biosphere. Äspö Hard Rock Laboratory and Outokumpu deep borehole are the prominent deep biosphere sites of the Fennoscandian shield. Lubin copper mine, in Poland, is also one site where the microbiome of the subsurface has been studied. The mechanisms by which these organisms adapt to such environments are studied extensively in this site. Organisms found here are mostly mesophilic in nature and can survive in high pH. The microbiome broadly consists of methanogens and sulfate reducers (Hallbeck and Pedersen 2012; Kotelnikova and Pedersen 1997; Rajala et al. 2015; Dziewit et al. 2015; Rajala and Bomberg 2017; Purkamo et al. 2017). Many ultradeep mines and gold mines in the African continent have been explored to study about the deep subsurface organisms. When native organisms from the samples were enriched under different conditions using specific medium or using supplements in the sample itself, a variety of organisms could be reported. Organisms belonging to archaeal and bacterial lineages which can sustain in extremes of temperature and pH were found to be prevalent in these environments. Methanogenic organisms were also reported (Lazar et al. 2017; Onstott et al. 2003; Kieft et al. 2005; Lin et al. 2006). An elaborate details about different organisms that are identified from these selected study sites based on cultivation-dependent studies has been provided in Table 8.2.

Table 8.2 Details of the selected terrestrial deep biosphere sites based on culture-dependent and enrichment-based studies

Area	Sample and depth	Medium	Temperature and pH	Organisms/taxa identified (isolation or via sequencing)	References
Asia: Chinese continental scientific drilling Project	Rock samples, 529–2026 m	Minimal medium M1 and FWA-Fe (III) medium	Thermophilic and alkaliphilic	*Proteobacteria*, *Bacteroidetes*, *Planctomycetes*	Zhang et al. (2005)
Asia: Mizunami Underground Research Laboratory, Central Japan	Groundwater, 1148–1169 m	Groundwater supplemented with different nutrients	Mesophilic and alkaliphilic	*Proteobacteria*, *Firmicutes*, *Brevundimonas* spp., *Thauera* spp.	Fukuda et al. (2010)
North America: Piceance Basin, Colorado	Sandstone, 856–2096 m	PTYG and TSA	Mesophilic and thermophilic	*Desulfotomaculum nigrificans*	Colwell et al. (1997)
North America: Antrim Shale	Sedimentary rocks, 302–520 m	DSMZ medium 141 and 318	Mesophilic	*Methanocorpusculum bavaricum*, *Methanomicrobiales*	Waldron et al. (2007)
North America: Lupin Au mine, Canada	Saline fracture water, 890–1130 m	Low nutrient marine broth/0.1× tryptic soy broth	Subpermafrost psychrophilic	*Desulfosporosinus*, *Halothiobacillus*, *Pseudomonas*	Onstott et al. (2009)
North America: Snake River Plain Aquifer	Basaltic core and fracture water, 63–134 m	R2A medium and nitrate mineral salts medium	Mesophilic and alkaliphilic	*Pseudomonas*, *Burkholderia*, *Brevundimonas*, *Acidovorax*, *Hydrogenophaga*, *Xanthobacter*, *Alcaligenes*, *Aurobacterium*, *Flavobacterium*, *Rhodococcus*, *Rhodobacter*, *Nocardia*, *Paenibacillus*, and *Micrococcus*	Lehman et al. (2004)
Europe: Swedish repository for spent nuclear fuel	Granitic groundwater, 112–978 m	Formulated medium for isolation of SRB, NRB, IRB, MRB, AA, AM, HA, HM	Mesophilic and alkaliphilic	*Methanosarcina*-like organisms, *Methanohalophilus*-related organisms, *Methanobacterium*	Hallbeck and Pedersen (2012)
Europe: Aspo hard rock laboratory	Granitic groundwater, 68–446 m	Groundwater-based medium	Mesophilic and alkaliphilic	*Methanobacterium*	Kotelnikova and Pedersen (1997)

(continued)

Table 8.2 (continued)

Area	Sample and depth	Medium	Temperature and pH	Organisms/taxa identified (isolation or via sequencing)	References
Europe	Fracture fluid, 500 m	Fracture fluid supplemented with methane or methanol	Mesophilic	*Desulfobulbus, Desulfobacterium, Desulfovibrio*, γ-proteobacterial group, methanotrophs	Rajala et al. (2015)
Europe: Lubin copper mine, Poland	600 m	Luria-Bertani medium, with required supplements	Mesophilic	*Pseudomonas, Brevundimonas* sp. LM17 and LM18, *Ochrobactrum* sp. LM19, *Paracoccus* LM20, *Sinorhizobium* sp. LM21, *Achromobacter* sp. LM16, *Psychrobacter* sp. LM26, *Stenotrophomonas*	Dziewit et al. (2015)
Europe: Outokumpu, Finland	180 and 500 m	Fracture fluid supplemented with methane or methanol	Mesophilic	*Betaproteobacteria*, *Clostridia*, *Bacteroidia* and *Anaerolineae*, *Gammaproteobacteria* (mainly genus *Pseudomonas*), with *Clostridia*, *Betaproteobacteria*, *Alphaproteobacteria* (*Rhodobacter*)	Rajala and Bomberg (2017)
Europe: Outokumpu deep drill hole	Fracture fluid, 967 m	Fracture water supplemented with different nutrients	Mesophilic	*Alphaproteobacteria*	Purkamo et al. (2017)
Africa: Hainich CZE	Carbonate/ siliciclastic rock, 8.5–69 m	Groundwater supplemented with different nutrients	Mesophilic	*Thaumarchaeota*, *Woesearchaeota*	Lazar et al. (2017)
Africa: Ultradeep mines (South Africa)	Rock, air, service water, 3200 m	Sulfolobus medium Fe(III)-reducing media	Mesophilic and thermophilic	*Proteobacteria*	Onstott et al. (2003)
Africa: Gold mine, Witwatersrand Basin (South Africa)	Groundwater, 3100 m	Basal salts medium amended with various combinations of electron donors and electron acceptors	Thermophilic and alkaliphilic	*Alkaliphilus crotonatoxidans, Alkaliphilus transvaalensis*	Kieft et al. (2005)
Africa: Mponeng gold mine (South Africa)	Groundwater, 2800 m	Groundwater supplemented with different nutrients	Thermophilic/ alkaliphilic	*Proteobacteria*, *Firmicutes*, *Methanobacteria*	Lin et al. (2006)

8.4 Cultivation-Independent Studies

8.4.1 Why Culture-Independent Studies Are Necessary?

Even two decades back, scientists were more eager to cultivate bacteria from the environment and study their characteristics. This was one of the key steps to understand their ecological role and biogeographic pattern in different environments. Designing media and mimicking environmental conditions was one of the important tasks for the microbial ecologist. Sometimes, it would take years to understand the key nutrient and the conditions required for isolating a particular type of microorganism. Question about limits of life in different extremes was one of the important topics that stormed the scientific community. In deep biosphere, which is one of the toughest places on our planet, exploration of deep life and other deep life initiatives as a part of different Integrated Ocean Drilling Program (IODP) and International Continental Scientific Drilling Program (ICDP) surfaced up and later became key components of these initiatives. Even one of the greatest initiatives for deep life research was part of the decadal goals set by Deep Carbon Observatory (https://deepcarbon.net). Cultivable approaches for different deep biosphere studies often took time and gathered limited knowledge about adaptability and sustenance of life in such extremes. Among different questions that remained unanswered or partly answered, the following ones are the most important with respect to deep life (Colwell and D'Hondt 2013; Kieft 2016):

(a) What are the processes that define the diversity and distribution of deep life?
(b) What are the environmental limits of life?
(c) How do the microorganisms in the deep subsurface interact with different global biogeochemical cycles?

Answering such questions becomes difficult and more challenging using cultivation-based approaches. Advent of metagenomics-based studies created unprecedented opportunities to investigate and understand the deep biosphere. Earlier, metagenomics studies were mainly focused on targeted gene sequencing using clone library approaches. Often this method took longer time but gave an overview of the structural and functional profiles of a community in such extremes. Other methods like denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) were developed to get a better impression of microbial community pattern in diverse environments (Schütte et al. 2008). Some of the main drawbacks of these methods were that it would take a huge number of sequencing of cloned targeted DNA which is often expensive, and dominant microorganisms are much more revealed as compared to rare microbiome. With the advent of sequencing technologies, next-generation sequencing (NGS) came in being which not only reduced the sequencing cost and time but also gave a better overview of the microbial community structure and function. Sequencing through NGS technologies can bring out the rarest taxa in a microbiome since the

depth of sequencing technologies has increased immensely. NGS also gave an additional advantage of massively parallel sequencing which saved a lot of time.

Application of NGS in deep biosphere study has been applied in two main ways to get an overview of the biodiversity. One of the two ways is through targeted DNA-based amplicon sequencing, whereas the other way is through shotgun metagenomics approach. Each of the methods has their own merits and demerits. Amplicon sequencing gives an overview of the overall microbial population with respect to particular amplified gene fragment. The most used marker gene for biodiversity study is through targeting hypervariable regions of the 16S rRNA gene. The data pool generated by amplicon sequencing is much smaller than that generated during shotgun whole metagenome sequencing and gives us an overview about the community from a single perspective. Since this method requires primer-based amplification through PCR, often the dominant microorganisms are revealed and biases are created for some primer sets used during sequencing. Shotgun whole-metagenome sequencing is much more robust and gives us a better assessment of the microbial community and function (binning and reconstruction). It not only helps to understand the possible biogeochemical cycles in the deep biosphere but also helps to predict probable interactions and behavior pattern among microorganisms in a community.

8.4.2 Microbial Ecology of Igneous Provinces

Igneous provinces are often characterized by low microbial biomass due to the oligotrophic nature of the rocks and associated environments. Knowledge about their functional potential is limited. Though studies in the subterranean igneous environments are limited, investigations in different seafloor basalts and surface environments of igneous provinces give us an overview about the microbial communities. The presence of Mn-oxidizing bacteria in basalts from Loihi Seamount and neutrophilic Fe-oxidizing bacteria in oceanic basaltic glass were reported by Edwards et al. (2003) and Templeton et al. (2005), respectively, whereas microbial communities from basaltic glasses of the Knipovich Ridge, Arctic, consisted mainly of heterotrophs and some chemolithotrophs (Thorseth et al. 2001). Iron-reducing bacteria were cultured from Arctic Ridge seafloor basaltic glasses, and the presence of other organisms belonging to the *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Actinobacteria*, and *Crenarchaeota* of unknown physiology were also reported (Lysnes et al. 2004). Microbial community diversity of two volcanic terrestrial glasses of Valafell and Dómadalshraun lava flow, Iceland, was mainly dominated by *Actinobacteria* followed by *Proteobacteria*, *Acidobacteria*, and *Cyanobacteria* (Kelly et al. 2010). In another report, dominance of *Proteobacteria* was found in another Dómadalshraun site, and dominance of *Actinobacteria* was observed at Hnausahraun site, Iceland (Kelly et al. 2011). *Betaproteobacteria* consisting of nonphototrophic diazotrophs such as *Herbaspirillum* spp. and chemolithotrophs such as *Thiobacillus* dominated the microbial communities of Fimmvörðuháls Lava Flow, Eyjafjallajökull, Iceland, but dearth of photosynthetic

groups possess a contrast to the microbial communities of older Icelandic lava flow (Kelly et al. 2014). Bacterial communities of hot, anoxic crustal fluids within Juan de Fuca Ridge flank subsurface basalt at boreholes U1362A and U1362B were represented by lineages of phylogenetically unique *Nitrospirae*, *Aminicenantes*, *Calescamantes*, and *Chloroflexi*, whereas less abundant archaeal community was dominated by unique, uncultivated lineages of Marine Benthic Group E, Terrestrial Hot Spring Crenarchaeotic Group, *Bathyarchaeota*, and relatives of cultivated, sulfate-reducing *Archaeoglobi* (Jungbluth et al. 2016).

Microbial diversity of crystalline granitic bedrock system was studied at Äspö Hard Rock Laboratory (HRL), Sweden, in which nitrate-, iron-, manganese-, and sulfate-reducing microorganisms along with acetogens and methanogens were suggested to be part of such anaerobic and oligotrophic environment (Pedersen et al. 1993; Hallbeck and Pedersen 2008). Microbial diversity of deep-granitic-fracture-water in Colorado was mainly represented by *Nitrosomonadales* in the oxic borehole, whereas dominance of anaerobic bacteria was observed in plugged borehole (Sahl et al. 2008). In the same study, sequences from 1740 m-deep granitic core were represented by *Proteobacteria* (primarily by *Ralstoniaceae*) and *Firmicutes*. In the Chinese continental scientific drilling project, 16S rRNA gene analysis revealed that *Proteobacteria* dominated the microbial community of ultra-high-pressure rocks, and most of the organisms were related to nitrate reducers from a saline, alkaline, and cold habitat (Zhang et al. 2005). Microbial communities in the deep crystalline rock system of Fennoscandian shield were represented by highly diverse group of bacterial and archaeal populations with versatile metabolic capabilities for hydrogen-driven carbon cycling, reduced carbon compound assimilation, and nutrient cycling (Nyyssönen et al. 2014). In contrast to the hydrogen-driven lithoautotrophic systems, Purkamo et al. (2015) reported dominance of carbon assimilation by heterotrophic groups like *Clostridia* in Outokumpu deep scientific drill hole.

8.4.3 Deep Biosphere Studies of Terrestrial Subsurface

The terrestrial deep biosphere of our planet consists of diverse habitat ranging from deep aquifer system, mines, caves, and other sedimentary and igneous provinces. Though different natural environments and man-made infrastructures are present, investigations in the deep subsurface are often restricted by inaccessibility of samples from deep environments. Scientific drillings are frequently required to study the deep biosphere at greater depths (Gold 1992). Different studies are conducted to investigate microbial ecosystems of the deep subterranean environment. Some of the major study locations are marked in Fig. 8.6. Most of the deep biosphere studies involved groundwater or fracture fluid samples. Study of rock-hosted microbiome of the deep terrestrial subsurface is limited (Fig. 8.6). Details of selected study sites marked on the world map include investigations of deep biosphere of four continents, viz., North America, Europe, Asia, and Africa.

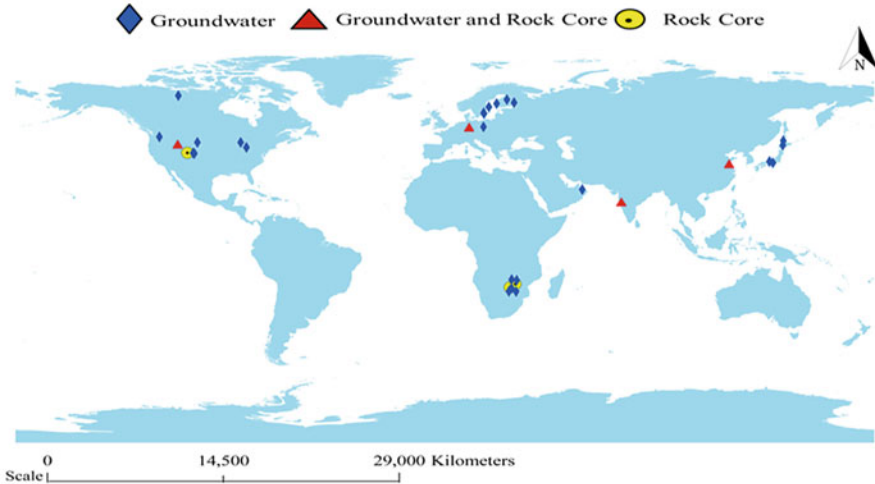

Fig. 8.6 Major subterranean deep biosphere sites from around the globe (map not to scale)

8.4.3.1 North America

There are several deep biosphere studies from different parts of North America. One of the first studies was done in the deep basaltic aquifer of Columbia River Basalt (CRB) where lithoautotrophic microbial ecosystem devoid of photosynthetic inputs was observed (Stevens and McKinley 1995). This was the first study to hypothesize hydrogen-driven ecosystem in subsurface province. Subsequent study in CRB reported the presence of sulfur-reducing bacteria (SRB) and metal-reducing bacteria from two deep anaerobic, alkaline aquifers (Fry et al. 1997). Microbial communities from deep low-biomass sandstone of Piceance Basin, Western Colorado, USA, were also explored where presence of anaerobic bacteria (mainly iron-reducing and fermentative bacteria) was reported (Colwell et al. 1997). Similar exploratory studies were done in basaltic aquifers of Snake River Plains (Newby et al. 2004; Lehman et al. 2004; O'Connell et al. 2003). These investigations revealed presence of both bacterial and archaeal members which included heterotrophs, methanotrophs, ammonia oxidizers, hydrogen oxidizers, iron reducers, propanotrophs, and phenol oxidizers. Exploration of methylotrophic and methanogenic communities in the subsurface sedimentary rocks of Antrim Shale suggested that local subsurface environment governed the microbial community structure (Waldron et al. 2007). Microbial community structure and functions were explored in different deep mine environments of North America, viz., Henderson mine (USA), Homstake mine (USA), and Lupin Au mine (Canada). One of the first extensive studies of deep biosphere in deep mine environments of North America is in Henderson mine located in Colorado (Sahl et al. 2008). This study revealed presence of *Firmicutes* and *Proteobacteria* where inorganic carbon fixation was proposed to be an important microbial metabolism. Iron-reducing *Gallionella* sp. was also observed in this subsurface environment. Later study in Henderson mine focused on N_2 fixation

and nitrification processes of the subsurface where presence of different genes involved in nitrogen cycling was correlated with NH_4^+ concentration and importance of NH_4^+ as an energy source was assessed (Swanner and Templeton 2011). Microbial community structure in Homstake Gold mine was dominated by proteobacterial members where distinct microbial communities in two different sites were observed (Rastogi et al. 2009). Investigation of subsurface microbiome at Lupin Au mine reported the presence of *Desulfosporosinus*, *Halothiobacillus*, and *Pseudomonas* as the dominant bacterial groups where sulfate reduction and sulfide oxidation via denitrification were found to be the most thermodynamically favorable processes (Onstott et al. 2009). Elaborate studies were also conducted regarding microbial community structure and function in Sanford Underground Research Facility (SURF). Bacteria were found to be more dominant over archaea in this subsurface environment where microorganisms are thought to derive energy from the oxidation of sulfur, iron, nitrogen, methane, and manganese (Osburn et al. 2014). Later detailed metagenomic study at SURF focused on energy and carbon metabolism where sulfate and nitrate/nitrite reduction were found to be the most common putative energy metabolism and energy-efficient Wood-Ljungdahl pathway was the most common autotrophic carbon fixation pathway (Momper et al. 2017).

8.4.3.2 Asia

Major deep biosphere studies in Asia covered parts of Japan, China, and Oman. Chinese Continental Scientific Drilling Project at Donghai, China, is one of the earliest and deepest (2026 m) explored subsurface biosphere studies in Asia (Zhang et al. 2005). Subsurface environment of this site was mainly dominated by proteobacterial members. *Bacteroidetes*, *Planctomycetes*, and Candidatus taxa were also observed. Presence of thermophilic, alkaliphilic, and iron-reducing bacteria was observed in the fluids, whereas rock-hosted microbiome harbored mesophilic and psychrophilic microorganisms.

In Japan, several studies were conducted in mine environments and established Underground Research Laboratories (URL). One of the first deep biosphere studies in Toyoha mine in Japan suggested presence of thermophilic SRB in the deep mine environment (Nakagawa et al. 2002). Later study in oligotrophic aquifer near Tono Uranium Mine, Japan, demonstrated the utility of $\Delta\ ^{13}C$ PLFA and $\Delta\ ^{14}C$ PLFA in understanding microbial carbon cycling in the deep subsurface environment (Mills et al. 2010). Carbon sources used by bacterial population in sedimentary versus igneous host rock were ascertained in this study. Another study on microbial diversity of deep subsurface fault-bordered aquifer in the Miocene formation suggested coexistence of methanogens and SRB (Shimizu et al. 2007). Two different aquifers were investigated in this study, where one of the aquifers was dominated by archaeal groups (sequence related to *Methanoculleus*), whereas the other aquifer was predominated by bacterial members such as *Bacteroidetes*, *Firmicutes*, and *Deltaproteobacteria*. Exploration of microbial diversity in ultra-deep granitic groundwater aquifer at Mizunami URL revealed that *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Firmicutes* were the major residents in this subsurface environment (Fukuda et al. 2010).

Later study at Mizunami URL suggested prominent shift in microbial diversity over different time periods (Ino et al. 2016). In the same study, NanoSIMS analysis was also conducted which confirmed the presence of active microbial population in the deep granitic groundwater. Extensive investigation on microbial community structure and function was also conducted at Horonobe URL where presence of diverse microbial lineages including phyla that did not have any cultivated representatives was reported (Hernsdorf et al. 2017). Majority of microorganisms in this ecosystem could metabolize H_2 via Ni-Fe hydrogenase and Fe-Fe hydrogenases, and it was postulated that these microorganisms could also catalyze carbon, nitrogen, iron, and sulfur transformations. Among different other deep biosphere investigations in Asia, exploration of microbial habitability in Oman hyperalkaline peridotite aquifers is an eminent one (Miller et al. 2016). Microbial investigation in gas-rich hyperalkaline fluids suggested that low-temperature H_2 and CH_4 generation, coupled with the presence of electron acceptors such as NO_3^- and SO_4^{2-}, drives the deep biosphere within Oman ophiolite. In India, extensive studies have been conducted to explore the subsurface microbial community structure and function of deep granitic-basaltic environments at different depths (60–1500 meters below surface) of Koyna-Warna region of Deccan traps (Dutta et al. 2018a). Metagenomic studies revealed distinct microbial communities residing across different subterranean provinces of Koyna-Warna region. Microbial diversity of the deep Deccan also suggested partitioning of interrelated microbial guilds on the basis of rock geochemistry where synergy was observed across different microbial classes (Dutta et al. 2018a, b).

8.4.3.3 Europe

The most studied location in Europe is the Fennoscandian shield which is present in the northern part of Europe. Several investigations of subterranean deep biosphere in this location have widened our knowledge in the field of deep biosphere. Äspö Hard Rock Laboratory (AHRL), Outokumpu deep borehole, and Olkiluoto are the prominent deep biosphere sites of the Fennoscandian shield.

One of earliest studies in AHRL assessed the diversity of methanogenic archaea and homoacetogenic bacteria (Kotelnikova and Pedersen 1997). In this study, it was postulated that deep granitic groundwater from AHRL is inhabited by autotrophic methanogens and acetogens, which may produce methane and acetate at the expense of subterranean H_2 and HCO_3^-. Another study at AHRL reported the presence of nitrate-reducing bacteria (NRB), iron-reducing bacteria (IRB), manganese-reducing bacteria (MRB), and sulfate-reducing bacteria (SRB) in the deep subsurface where methanogens and acetogens were also observed (Hallbeck and Pedersen 2008). One of the recent studies at AHRL reported an extensive investigation of three subsurface aquifers through metagenomic approach (Wu et al. 2015). Two of the major findings of this research are (i) phylogenetically distinct microbial community subsets were observed across different aquifers and (ii) microbial communities having small cell size also had a tendency to have smaller genomes than their closest sequenced relatives which might be due to physiological adaptation to life in highly oligotrophic deep biosphere groundwaters.

In the deep groundwater of Olkiluotu (OL), presence of NRB, IRB, SRB, MRB, acetogenic bacteria, and methanogens was observed (Haveman et al. 1999; Pedersen et al. 2008). It was found that at OL, fracture-filling minerals were a better indicator of microbial populations than was groundwater chemistry (Haveman et al. 1999). Results also suggested that anaerobic methane oxidation may be a significant process in subsurface groundwater of OL (Pedersen et al. 2008). A later study at OL focused on methanogenic and sulfate-reducing microbial communities of deep granitic groundwater (Nyyssonen et al. 2012). Higher abundance of *dsr*B was found in samples having higher SO_4^{2-} concentration, and SRBs were mainly affiliated to different orders of *Deltaproteobacteria*. Results imply that sulfate reduction, methanogenesis, and anaerobic methane oxidation may also take place in this environment. One of the recent deep biosphere studies at OL reported that microbial communities varied with depth, salinity gradient, and sulfate and methane concentrations (Bomberg et al. 2015). In this study, the highest bacterial diversity was observed in the sulfate-methane mixing zone (SMMZ), whereas archaeal diversity was highest in the lowest boundaries of SMMZ.

Among other sites in Europe, most number of studies have been conducted at Outokumpu Deep Borehole (ODB) located in Finland. One of the first studies at ODB explored the microbial diversity of the deep groundwaters where microbial diversities were found to be varying as a function of depth and microbial community composition was linked to geochemistry of groundwater (Itavaara et al. 2011). Similar results were obtained in the subsequent studies at ODB (Purkamo et al. 2013; Nyyssonen et al. 2014). In 2014, Nyyssonen et al. reported the presence of chemoheterotrophic, chemolithoheterotrophic, thiosulfate-reducing, sulfite-reducing, and fermentative groups in the deep groundwaters of ODB where *Proteobacteria*, *Firmicutes*, and *Tenericutes* were found to be the most abundant bacterial phyla, whereas hydrogenotrophic methanogens were the most abundant archaeal groups. Another important study at ODB reported the prevalence of heterotrophic microbial groups (such as *Clostridium*) throughout the drill hole water column which was studied using marker genes for carbon assimilation, methane production, and methane consumption (Purkamo et al. 2015). Subsequent study explored the responses of microorganisms (residing in deep groundwater of ODB) to C-1 compounds (Rajala et al. 2015; Rajala and Bomberg 2017). It was found that dormant microbes from the deep became active in presence of C-1 substrates and suitable conditions.

Some of the other studies in Europe include an investigation on the role of plasmids in adaptation of bacteria in subsurface environment (Lubin copper mine, Poland); exploration of microbial diversity at high-pressure deep subsurface environment (Pyhäsalmimine, Finland); examining the microbial diversity and functionality of archaeal, bacterial, and fungal population of deep Archaean bedrock fracture aquifer (Romuvaara, northern Finland); and assessing the archaeal diversity of subsurface carbonate−/siliciclastic-rock environment (Hainich CZE, Germany) (Dziewit et al. 2015; Miettinen et al. 2015; Lazar et al. 2017; Purkamo et al. 2018).

8.4.3.4 Africa

Microbial ecology of deep biosphere of Witwatersrand basin in South Africa is widely explored. Ultra-deep gold mines in this region have provided an easy access to the deep subsurface. One of the first studies in the deep gold mines of Witwatersrand basin focused on the archaeal diversity (Takai et al. 2001). In this study, novel archaeal lineages, viz., SAGMCG and SAGMEG, were reported for the first time. Later studies focused on overall microbiome of the deep subterranean provinces of Witwatersrand basin. Comparative analysis of microbial diversity across subsurface rock, service water, and air of a 3.2 km deep gold mine was conducted to analyze the chance of contamination, and it was found that contamination of rock cores by service water was negligible accounting for less than 0.01% contamination (Onstott et al. 2003). A later study in similar environment reported presence of different H_2 generating processes, namely, serpentinization, oxidation of ferrous silicate minerals, and radiolysis of water which could fuel the microbial community in the deep (Kieft et al. 2005). The findings of this study have significance for other deep subsurface environments on Earth and possibly for those of other planetary bodies as well. Presence of *Firmicutes*, *Proteobacteria* and *Euryarchaeota* was also reported from ultra-deep gold mines where thermophilic sulfate-reducing *Firmicutes* were observed which could sustain on geologically produced SO_4^{2-} and H_2 generated in the deep (Borgonie et al. 2015; Gihring et al. 2006; Lin et al. 2006). One of the investigations in similar subterranean environment of Witwatersrand basin focused on nitrogen cycling in the deep where an array of genes related to nitrogen cycling were observed from metagenomic analysis and evolutionary relationship between surface and subsurface genes of microorganisms was assessed which suggested that subsurface habitats have preserved ancestral genetic signatures (Lau et al. 2014). With the advancement of technology and analytical tools, studies in South African subcontinent became more intricate and informative. One of the eminent and recent studies in deep environments of Witwatersrand basin focused on metabolic networks and trophic structures of microbial communities using metatranscriptomics, metaproteomics, and thermodynamic modeling (Lau et al. 2016). This study revealed that deep subsurface community in this oligotrophic environment is dependent on syntrophy where sulfur-dependent autotrophic denitrifiers are the dominant group. One of the other recent studies focused on carbon metabolism at Precambrian continental crust of Tau Tona gold mine where the energy-conserving Wood-Ljungdahl pathway was found to be the most abundant carbon fixation pathway (Magnabosco et al. 2016). This study also revealed that *Firmicutes* and *Euryarchaeota* were the most abundant members in the metagenome which is in line with previous studies in similar environment (Gihring et al. 2006; Lin et al. 2006) (Fig. 8.6).

8.5 Techniques for Sampling the Subsurface

Deep drilling is required either from the surface or from a pre-existing subsurface site, e.g., in deep mines for obtaining deep subsurface samples for microbiological as well as geological investigations (Kieft 2016; Wilkins et al. 2014). Selection for the appropriate drilling and coring methods is decided on the basis of geological formation(s) to be sampled and also on the scientific aims of the project. Basically, there are three types of drilling techniques, namely, (1) hollow-stem augering, (2) cable-tool drilling, and (3) rotary drilling using a drilling fluid (for acquiring the sample from deeper depth and crystalline environments), which are used. They use portable drill rigs or larger rigs for deeper drilling, which are assembled on site (Kieft 2010). The underground mining industry uses small drill rigs that can be deployed in the limited space of mine tunnels to drill through rock to depths of 3000 m or more (Sahl et al. 2008). Since drilling is innately quite messy, detection and removal of contamination from the subsurface samples have been a necessity for characterizing the microbiology of these habitats (Phelps et al. 1989; Kieft 2016). Soil, atmospheric, and human-associated microorganisms, material from overlying formations sloughed off in the borehole, chemical contamination from the atmosphere (including O_2), hydrocarbons used for lubrication, etc. can be potential contaminants during deep drilling (Kieft et al. 2007). Quite some time back, Pedersen et al. (1997) had reported the presence of *Acinetobacter*, *Methylophilus*, *Pseudomonas*, and *Shewanella* in drilling-related equipment. Since then, there have been much advancement in the drilling, coring, and sampling technologies such that samples can be extracted aseptically from deeper environments (>3 km depth) (Lin et al. 2006; Moser et al. 2005; Onstott et al. 1998). Techniques have been devised for aseptic handling of samples and their proper storage (in freezing conditions) in the absence of oxygen to preserve oxygen-sensitive anaerobes (Kieft 2016). Online gas analyses can be performed onsite during scientific drilling to recognize biologically active zones (Erzinger et al. 2006).

Another important aspect of deep drilling is the use of drilling fluid (gaseous, liquid, slurry, or foam) during sampling from deep subsurface for intact recovery of deep subsurface rock cores (Kieft et al. 2007). Drilling fluids lubricate and cool the drill bit and maintain the hydrostatic pressure during the drilling operations (Kieft 2016). These fluids can be problematic, especially when drilling fluids with organic additives (bentonite based) are used (Struchtemeyer et al. 2011). These drilling fluids are one of the most prominent sources of microbial contamination in deep subsurface study (Kieft et al. 2007; Kieft 2010). Drilling fluid is expected to possess microorganisms that originate from the surface and are carried to depth during drilling operations. Solute and particulate tracers which include fluorescent dyes, LiBr, and perfluorinated hydrocarbons (Table 8.3) can be added to the drilling fluid. Later, the subsurface samples can be quantified by different analytical methods to detect the presence of these tracers in the subsurface samples in order to determine the extent of contamination from drilling fluids (Phelps et al. 1989; Kieft 2016). It may be quite possible that the subsurface samples are tracer-free, but there might be still a chance of microbial contamination. Hence, the microbial communities in the

Table 8.3 Tracers used for contamination assessment in subsurface drilling

Sl. No.	Nature and area of sample	Type of drilling fluid	Type of tracer	References
1.	North hydrothermal field, mid Okinowa trough (IODP 331), sand, gravel, and clay	Guar gum (0.8%) or seawater gel mud + 5% bentonite + 0.1% sodium hydroxide + 0.1% lime + barite + surface seawater containing guar gum mud	Perfluoromethylcyclohexane (C_7H_{14}) [PFT], 1 mg L^{-1} Fluorescent microspheres (0.5 μM diameter)	Yanagawa et al. (2013)
2.	Waikato area, North Island, New Zealand (Cenozoic sediments)	Bentonite + a small quantity of Pac-R polymer + water	Fluorescent microspheres (8.4%)	Kallmeyer et al. (2006)
3.	Western Pacific during ODP leg 185, Pigafetta Basin, South China Sea (sediments and igneous rocks)	Surface seawater	perfluoromethylcyclohexane 1 mg L^{-1}. [PFT] fluorescent microspheres, 10^{10} spheres mL^{-1} (0.5 μM diameter)	Smith et al. (2000)
4.	The Mineral Park mine, Arizona, igneous rocks	Sacramento Valley groundwater + sodium hypochlorite (60 mg L^{-1})	Fluorescent microspheres	Lehman et al. (2001)
5.	The Savannah River plant (SRP), North Carolina (sediments)	Bentonite	Potassium bromide (900 mg L^{-1}), rhodamine (20 mg L^{-1})	Phelps et al. (1989)
6.	Deep anaerobic aquifer of Atlantic coastal plain of South Carolina, (Myrtle beach and Florence) (Subsurface sediments)	Local groundwater	Barium (10 mg L^{-1}) Fluorescent microspheres, 10^5 spheres mL^{-1} (1 μM diameter)	Chapelle and Lovely (1990)
7.	ODP leg 201 (subsurface sediments)	Seawater	Perfluorocarbon tracers (PFTs) (1 mg L^{-1}) Fluorescent microspheres 10^{10} spheres mL^{-1} (0.5 μM diameter)	House et al. (2003)
8	Precambrian granitic bedrock in SE Sweden	Groundwater	Fluorescein (500 mg L^{-1}) in water	Pedersen and Ekendahl (1990)
9	Äspö HRL, Baltic coast, Sweden	Groundwater	Fluorescent dye, uranine	Pedersen et al. (1997)
10	Opalinus clay, Switzerland	Compressed air (up to 9 m and pure N_2 for the next 6 m) was used for drilling	Fluorescent microspheres 10 mL of 3.5×10^{11} particles mL^{-1} (0.4 ± 0.01 μM diameter)	Gascoyne et al. (2007)

11	Ashfall tuff samples Rainier Mesa, Nevada test site (deep subsurface rocks)	Not mentioned	Lithium bromide in DF (26 mg L^{-1})	Haldeman et al. (1995)
12	Lake Towati sediment	Towati lake water	SPL-N fine grind fluorescent pigment Dispersion (DayGlo, Cleveland, OH) [pigment content 45%, size 0.25–0.45 μM] – 1×10^9 particles per mL drilling fluid	Friese et al. (2017)
13	Lake Chalco	Chalco lake water	A whitish tracer with light blue fluorescence under UV Excitation (day Glo SPL-594 N, RADGLO AFN-09) 1×10^9 particles per mL drilling fluid	Friese et al. (2017)
14	Natural gas wells, Texas	Bentonite (maximum) + cellulose + nut hulls + cedar fiber + xanthan gum + barite + lignosulfonate	Not mentioned	Struchtemeyer et al. (2011)

drilling fluid and in the subsurface rock cores can be analyzed by 16S rRNA gene-based microbial diversity analysis and compared as a further test for drilling-induced contamination (Miteva et al. 2014; Dong et al. 2014; Yanagawa et al. 2013). Subsurface rock cores can be tested for the presence of different allochthonous hydrocarbons that may be derived from the drilling equipment or drill additives (Kallmeyer et al. 2006). Drilling fluids can support the growth of extremely high densities of microbes, e.g., 10^8 cells ml^{-1} (Beeman and Suflita 1989; Kieft et al. 2007). Coker and Olumagin (1995) obtained different bacterial and fungal genera in drill cuttings, viz., *Staphylococcus*, *Acinetobacter*, *Serratia*, *Clostridium*, *Nocardia*, *Bacillus*, *Actinomyces*, *Micrococcus, Pseudomonas, Penicillium*, *Fusarium*, etc. Miteva et al. (2014) reported the presence of microorganisms mostly found in crude oil- or hydrocarbon-contaminated environments (hydrocarbon-degrading *Firmicutes* and other bacterial genera *Pseudomonas*, *Acinetobacter*, *Massilia*, *Paracoccus*, *Agrobacterium*, etc.) in the hydrocarbon-based drilling fluid (Estisol 240 and Coasol) used during NEEM Greenland ice core drilling project. Presence of *Betaproteobacteria* and *Gammaproteobacteria* and *Bacteroidetes* in guar gum- and seawater gel-based (bentonite plus sodium hydroxide) drilling fluid was reported during deep-sea drilling and coring by the D/V *Chikyu* (IODP expedition 331 and *Chikyu* shakedown expedition CK06–06) (Yanagawa et al. 2013; Inagaki et al. 2015). Interestingly, some researchers have also reported drilling fluid as carriers of deep subsurface microbial communities (Struchtemeyer et al. 2011; Zhang et al. 2006; Masui et al. 2008). Hence, there is a possibility that drilling fluids may contain signatures of subsurface microbial community.

A major problem encountered during deep subsurface research is "postcore extraction contamination" from laboratory reagents (i.e., extraction kits, Taq polymerase, or buffers) (Salter et al. 2014). It is extremely indispensable to maintain controls at each and every level of coring and postcoring processes. These controls must be analyzed for their microbial diversity to further distinguish between contaminants and subsurface microbial communities. Postcoring laboratory controls (reagent blanks, etc.) have been analyzed for the presence of potential contaminants, and a comprehensive review on this aspect has been already published (Sheik et al. 2018; Salter et al. 2014). *Betaproteobacteria* and *Gammaproteobacteria* were mostly encountered bacterial classes in the laboratory controls followed by *Actinobacteria*, *Alphaproteobacteria*, *Firmicutes*, and *Bacteroidetes*. *Pseudomonas*, *Propionibacterium*, *Acinetobacter*, *Ralstonia*, and *Sphingomonas* were the major genera found in laboratory blanks (Sheik et al. 2018 and reference therein). Advanced computational techniques have been developed that enable us to identify and filter out the "contaminant microbial populations" from the deep subsurface sequences (Jørgensen and Zhao 2016; Labonté et al. 2017; Reese et al. 2018; Sheik et al. 2018, and reference therein). But, removal of these contaminant microbial communities must be done with utmost care as it may remove some taxonomically novel microorganisms present in deep subsurface. Also, there have been many reports of taxonomically similar groups present in surface as well as deep subsurface (Struchtemeyer et al. 2011; Zhang et al. 2006; Yanagawa et al. 2013; Moser et al. 2005; Gihring et al. 2006; Davidson et al. 2011). Hence, identification of

contaminants from deep subsurface microbiological studies becomes a separate and an important area of research. A database can be developed for the microbial communities obtained from drilling fluids and controls encountered during deep drilling and postcoring processes to sort out the true representatives of the deep biosphere from imposters represented by contaminants (Sheik et al. 2018).

8.6 Applications of Deep Subsurface Research

The deep biosphere offers huge potential for the discovery of various new aspects of life, and important revelations are made with each and every new opportunity to probe the subsurface (Kieft 2016; Kallmeyer et al. 2012; McMahon and Parnell (2013). The deep subsurface research has various applications starting from hazardous waste disposal (nuclear wastes), CO_2 sequestration, and extraction of various metabolites from deep subsurface extremophiles (extremozymes and extremolytes) for biotechnological purpose. This section briefly discusses various applications of deep subsurface research.

8.6.1 Deep Subsurface as Nuclear Waste Repositories

Deep boreholes drilled through the Earth's crust are an efficient disposal source for high-level nuclear wastes. This concept (deep borehole disposal, DBD) has been around for about 40 years (Schwartz et al. 2017). Researchers from the United States (US), the United Kingdom (UK), and Sweden have periodically examined DBD as a potential alternative to a mined repository (Schwartz et al. 2017 and reference therein). One of the biggest advantages of DBD as identified by researchers from Sweden is waste deposited in deep boreholes at 3–5 km depth would exist in a moderately torpid, density-stratified hydrogeologic arrangement as compared to more active shallower flow systems in a mined repository. Also, future glaciation, earthquakes, or human intrusion would be much less likely to disturb the waste at those depths (Ahall 2007). It is a secure way of disposing nuclear materials, since the deep depth of disposal in a small borehole provides a "formidable physical barrier" to the future retrieval of materials for spiteful purposes (Hippel and Hayes 2009). Additionally, there is no release of radionuclides through groundwater (Beswick 2008). A concept of DBD developed by the US Department of Energy (DOE) envisages disposal of radioactive waste in boreholes up to 5 km deep, completed in crystalline basement rock in containers and bentonite, concrete, and other materials would seal the upper 3 km of each borehole to isolate the waste from the biosphere (Brady et al. 2012). Utilization of granite and other crystalline rocks is advantageous since layers of argillaceous rocks at depth tend to be relatively unfractured, usually providing a natural barrier to groundwater flow, and these rocks have very high mechanical strength and they might resist borehole deformation during deep drilling (Brady et al. 2009).

8.6.2 Deep Subsurface CO_2 Sequestration

CO_2 can be stored in the deep subsurface in different types of formations. Since the last 40 years, CO_2 has been injected for improved oil recovery mainly in the USA and Canada (Firoozabadi and Cheng 2010; Benson and Cole 2008). Currently, 70,000 tons of CO_2 is injected worldwide per day for enhanced oil recovery (EOR). CO_2 can be even stored in underground depleted oil and gas reservoirs (Bouquet et al. 2009). Another well-accepted method for geological CO_2 sequestration is its storage in deep saline aquifers, because saline aquifers have larger storage capacities than other geological formations. Different trapping mechanisms include geological trapping, hydrodynamic trapping, and geochemical trapping (solubility trapping and mineral trapping). Mineral trapping which involves mineralogical reactions between dissolved CO_2 and formation rock is safer and more economical in the long term. Interactions among rock, water, and CO_2 initiated in the aquifer with CO_2 injection play a vital role in CO_2 sequestration in saline aquifers (De Silva et al. 2015). This process is extremely slow and can be made faster using deep subsurface microorganisms which harbors enzymes to aid the process. Even the injected CO_2 can be converted to methane by methanogens harboring in the deep subsurface (Gniese et al. 2013). Mu et al. (2014) displayed the alteration in microbial diversity as well and metabolism due to CO_2 injection in the geo-sequestration experiment at 1.4 km-deep Paaratte Formation of the Otway Basin, Australia. A general shift from *Firmicutes* to *Proteobacteria* was observed in the groundwater before and after CO_2 injection in the aquifer. Microbial reactions might have some favorable and unfavorable effects on CO_2 sequestration in deep boreholes (Ménez et al. 2007). Hence, it is extremely important to deduce the microbiology as well as the geochemistry of the deep borehole site before its use for CO_2 injection (Mu et al. 2014; De Silva et al. 2015).

8.6.3 Deep Subsurface as a Source of Novel Bioactive Compounds

The Earth's deep continental crust has geologically varied morphology with extreme conditions (temperature, pressure, pH, etc.) which makes it almost impossible for life to survive (Fredrickson and Balkwill 2006). Nevertheless, it is long been known that "deep subsurface" of the Earth hosts a diverse array of ecosystems which harbors a diverse population of extremophilic microbial life (Whitman et al. 1998; McMahon and Parnell 2013; Kieft 2016). These extremophiles harbor many novel bioactive compounds (extremozymes and extremolytes) which have potential applications in industries to produce biotechnologically important products in a cost-effective manner (Coker 2016). The most notable example is DNA polymerases obtained from thermophiles *Thermus aquaticus*, *Pyrococcus furiosus*, and *Thermococcus litoralis*, also known as Taq (Tindall and Kunkel 1988), Pfu (Lundberg et al. 1991) and Vent (Mattila et al. 1991), respectively. These extreme microbes are also known producers of extremozymes such as proteases and lipases, combined with the glycosyl hydrolases, which account for more than 70% of all enzymes sold

(Li et al. 2012). *Thermoanaerobacterium saccharolyticum* have shown tremendous applications in producing large quantities of biofuel (ethanol) and minimizing other side reactions/products (Basen et al. 2014). These thermophiles are even utilized in the production of hydrogen through anaerobic fermentation and hydrogenases. *Acidithiobacillus*, *Ferroplasma*, *Sulfolobus*, and *Metallosphaera* are widely utilized in biomining (removal of insoluble metal sulfides or oxides by using microorganisms) (Podar and Reysenbach 2006; Vera et al. 2013). Extremophiles are producers of a host of antibiotics, antimicrobial peptides (diketopiperazines), antifungals, and antitumor molecules (Littlechild 2015). Commercial success of DNA polymerase, enzymes, biofuels, and biomining obtained from extremophilic microorganisms proves that these extremophiles and their metabolites (primary and secondary) have an extensive foothold in biotechnology. New high-throughput technologies are the need of the day to produce most extremophiles/extremolytes on a large scale required by industrial processes (Coker 2016).

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9 Marine Fungal Diversity: Present Status and Future Perspectives

V. Venkateswara Sarma

Abstract

Fungal diversity in marine habitats varies with the techniques adopted. The processing of water and soil samples in artificial media on Petri dishes results in those similar to terrestrial environments, whereas direct examination of decaying plant substrata results in litter fungi mostly belonging to ascomycetes. With the advent of molecular techniques and retrieval of common soil fungi from deep-sea environments, it is now believed that the definition of marine fungi hitherto was narrow, and hence, the scope and definition of what is a marine fungus need to be expanded. Till 2009, there were 530 marine fungi that were reported, but after broadening the definition of marine fungi, this number has risen to 1112 species in 472 genera by 2015. The list included marine-derived fungi, which are now considered as marine fungi. The present number of marine fungi stands at 1206. Halosphaeriales belonging to *Ascomycota* is the most speciose order. Marine fungi are taxonomically diverse, though they may be physiologically or ecologically a defined group. Molecular sequence studies also reveal that marine environments comprise a large diversity of forms and lineages, including chytrids, filamentous hyphal forms, and multicellular forms. Ecologically, marine fungi play saprophytic and parasitic roles. A number of bioactive compounds have been reported from marine fungi which have therapeutic potential, including antimicrobial, antioxidant, anticancer, and various other disease states. Marine fungal diversity in the light of molecular inputs and their role in human welfare are discussed in this chapter.

Keywords

Culture-dependent · Culture-independent · Ecology · Enzymes · Bioactive compounds · Biodiversity · Biotechnology · Marine fungi · Molecular diversity

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9.1 Introduction

The known fungi are only around 100,000, of which *Ascomycota* and *Basidiomycota* (Dikarya) constitute 96% (Kirk et al. 2008), while the estimate has been 1.5 million (Hawksworth 1991, 2001) or up to 5.1 million species (O'Brien et al. 2005; Blackwell 2011; Taylor et al. 2014). Our knowledge on fungal diversity is still scanty because attempts to generate evolutionary relationships within and among major fungal lineages, outside of crown groups, have been hindered by inadequate taxon sampling (Picard 2017). Marine fungi constitute only a meagre 1% of described fungal species and are still poorly characterized (Jones 2011; Jones et al. 2015). Most of the studies on marine fungi were conducted from coastal habitats including driftwood, mangrove substrata, seaweeds, etc. for direct examination method or culturing of water, sediment, and sea foam (Kohlmeyer and Kohlmeyer 1979; Hyde and Sarma 2000). The results from these studies show that (1) most of the fungi belonged to Dikarya, with *Ascomycota* dominating in Dikarya, and (2) these fungi are localized to coastal habitats where organic matter is readily available (Kohlmeyer and Kohlmeyer 1979; Hyde et al. 2000; Picard 2017). The low diversity and abundance of marine taxa in open seawaters made Kohlmeyer and Kohlmeyer (1979) to opine that open oceans are by and large a "fungal desert." Recent culture-independent methods, such as environmental cloning and next-generation sequencing, reveal a substantially unknown fungal diversity in different habitats that belong not only to known fungal lineages but also to several entirely new clades that were hitherto not known (Picard 2017). A large number of these undescribed taxa in a diverse fungal tree are termed as "dark matter fungi" (DMF) (Grossar et al. 2016). However, a great majority of them belong to zoosporic fungi such as *Blastocladiomycota*, *Chytridiomycota*, *Cryptomycota*, *Neocallimastigomycota*, the genus *Olpidium*, and the previous *Zygomycota* lineages (Picard 2017). Many of these groups of fungi are microscopic and have fastidious nutritional requirements, thus making their isolation difficult. In fact, the newly described phylum *Cryptomycota* was introduced based on phylotypes recovered solely from environmental surveys (Jones et al. 2011). Besides providing clues about new taxa among existing terrestrial and freshwater habitats, the culture-independent methods also give clues about early divergent branches of fungal tree of life (Richards et al. 2012; Richards et al. 2015). In the past two decades or so, more than 1000 bioactive compounds have been reported from marine fungi (inclusive of marine-derived fungi). These aspects in addition to the role of marine fungi in environment are discussed in this chapter.

9.2 Diversity

Kohlmeyer and Kohlmeyer (1979) defined marine fungi to be "obligate marine fungi" if they grow and sporulate exclusively in a marine or estuarine habitat and "facultative marine fungi" if they occur in freshwater or a terrestrial milieu, being able to grow and also possibly sporulate in the marine environment. Marine fungi were earlier studied predominantly using two techniques. They are (1) direct

Table 9.1 Numbers of marine fungi and their increase at different points of time

Group	Kohlmeyer and Kohlmeyer (1979)	Kohlmeyer and Volkmann-Kohlmeyer (1991)	Hyde et al. (2000)	Jones et al. (2009)	Jones et al. (2015)
Total no. of marine fungi	106/209[a]	161/321	235/444	321/530	472/1112
Ascomycetes	62/149	115/255	177/360	251/424	352/805
Basidiomycetes	4/4	5/6	7/10	9/12	17/21
Anamorphic fungi	40/56	41/60	51/74	61/94	26/43
Chytridiomycetes	–	–	–	–	13/26
Yeasts	–	–	–	–	61/213

[a]Numbers in numerator indicate genera and denominator indicate species; – denotes not included

examination method and (2) isolation on artificial solid agar media. While the first technique yields typical marine fungi, the second one results in retrieval of fast-growing fungi (e.g., species belonging to *Aspergillus*/*Penicillium*), which are usually found in terrestrial environments and are known as "marine-derived fungi." Jones et al. (2009) compiled known marine fungi and listed 530 species which are mainly based on the former technique. Subsequently, Jones et al. (2015) updated the number to 1112 species belonging to 472 genera, including *Chytridiomycota*, marine yeasts, and marine-derived fungi in addition to the usually accepted filamentous higher marine fungi (Table 9.1). This number is based on the fungi retrieved involving both the abovementioned techniques with a broader definition of marine fungi. Pang et al. (2016) offered a changed definition for marine fungi as "any fungus that is recoverable repeatedly from marine habitats due to: (1) its ability to grow and/or sporulate (on substrata) in marine environments; (2) formation of symbiotic relationships with other marine organisms; or (3) its ability to adapt and evolve at the genetic level or be metabolically active in marine environments." Among the "marine-derived fungi," Jones et al. (2015) have chosen only those fungi that occurred repeatedly from marine environment in their revised list of marine fungi and did not consider those encountered once or twice. The present number of marine fungi stands at 1206 (Pang and Jones 2017). While Jones (2011) estimated that there could be 10,000 marine fungi, Richards et al. (2012) consider that marine fungi are low in both diversity and abundance.

9.3 Diversity of Culturable Marine Fungi

In the past, studies on marine fungi were mostly based on direct examination of the natural samples for the presence of reproductive propagules to identify them (Hyde and Jones 1988; Sarma and Vittal 2001). Few attempts have been made to isolate the obligate marine fungi (Jones and Hyde 1988; Raghukumar et al. 1994). Isolation of cultures is important and a prerequisite for various studies including physiological, biotechnological, and ecological aspects. In the past one decade, considerable

progress has been made on molecular phylogenetic studies of obligate marine fungi (Suetrong et al. 2009, Sakayaroj et al. 2011). While obligate marine fungi could be isolated following single-spore isolation technique for molecular analyses, the agar plate technique directly results in the isolation of "marine-derived fungi."

In a monographic account of the marine fungi, Jones et al. (2015) have accepted 1112 species of marine fungi in 472 genera, of which 805 species in 352 genera belonged to *Ascomycota*, 21 species in 17 genera to *Basidiomycota*, 26 species in 13 genera to *Chytridiomycota*, 3 species in 2 genera to *Zygomycota*, 1 species in 1 genus to *Blastocladiomycota*, 43 species in 26 genera to asexual filamentous fungi, and the remaining being marine yeasts (138 spp. in 35 genera of *Ascomycota*, 75 spp. in 26 genera of *Basidiomycota*). These 1112 species belong to 129 families and 65 orders. With 141 species in 59 genera, the *Halosphaeriaceae* numerically is still the leading family of marine fungi. Among the filamentous fungi, *Aspergillus* with 47 species and *Penicillium* with 39 species are the largest represented genera, while *Candida* is the most speciose (64 species) among yeasts.

The number of new marine fungi described has been increasing with 1206 as the updated number (Pang and Jones 2017). Earlier, it was thought that driftwood supports the highest diversity of marine fungi (Hyde and Sarma 2000). Subsequently, however, it has been found that mangroves also equally support a rich diversity of marine fungi (Hyde and Sarma 2000; Sarma and Hyde 2001; Jones and Alias 1997). Large tracts of mangrove forests are yet to be fully explored for marine fungi. On the other hand, several new fungi are being added to the list of marine fungi from marine environments wherever research is undertaken, particularly from mangroves (Devadatha et al. 2018a, b, c; Abdel-Wahab et al. 2018).

In addition to the diversity of obligate marine fungi increasing as newer sites and substrates are explored, the number of "marine-derived fungi" (that require agar plate studies) also has been increasing. The cultivation-dependent as well as molecular studies show that marine macrobes, including those that are isolated from algae and sponges, are a great source of fungi (Debbab et al. 2010).

Marine fungi thrive on a wide range of substrata such as wood, prop roots, pneumatophores, roots and putrefying leaves of mangroves, drift wood, intertidal grasses, living as well as dead animals, guts of crustaceans, sediments, soils, algae, corals, calcareous tubes of mollusk shells, etc. (Kohlmeyer and Kohlmeyer 1979; Hyde et al. 1998). Several marine fungi sporulate on sand grains (arenicolous) or other hard calcareous materials such as corals (Jones and Mitchell 1996).

More than 80% of marine fungi belong to *Ascomycota*, followed by other groups (Jones and Alias 1997; Sarma and Hyde 2001; Sarma and Vittal 2001) (Figs. 9.1 and 9.2). Ascomycetes apparently have evolved to take full advantage of aquatic habitats with their microscopic fruit bodies and spores that have appendages or mucilaginous sheaths that may aid in dispersal and attachment, thus enabling them to withstand fluctuating saline conditions (Hyde and Lee 1995; Jones and Alias 1997). Among the obligate marine fungi are the three basic groups, namely, (i) *Halosphaeriaceae* that have truly unique aquatic representatives, (ii) the marine unitunicate ascomycetes with terrestrial counterparts, and (iii) the marine *Dothideomycetes* with terrestrial and freshwater counterparts (Jones 2000). Within *Halosphaeriaceae*, two groups could be recognized: one that has both freshwater and marine genera

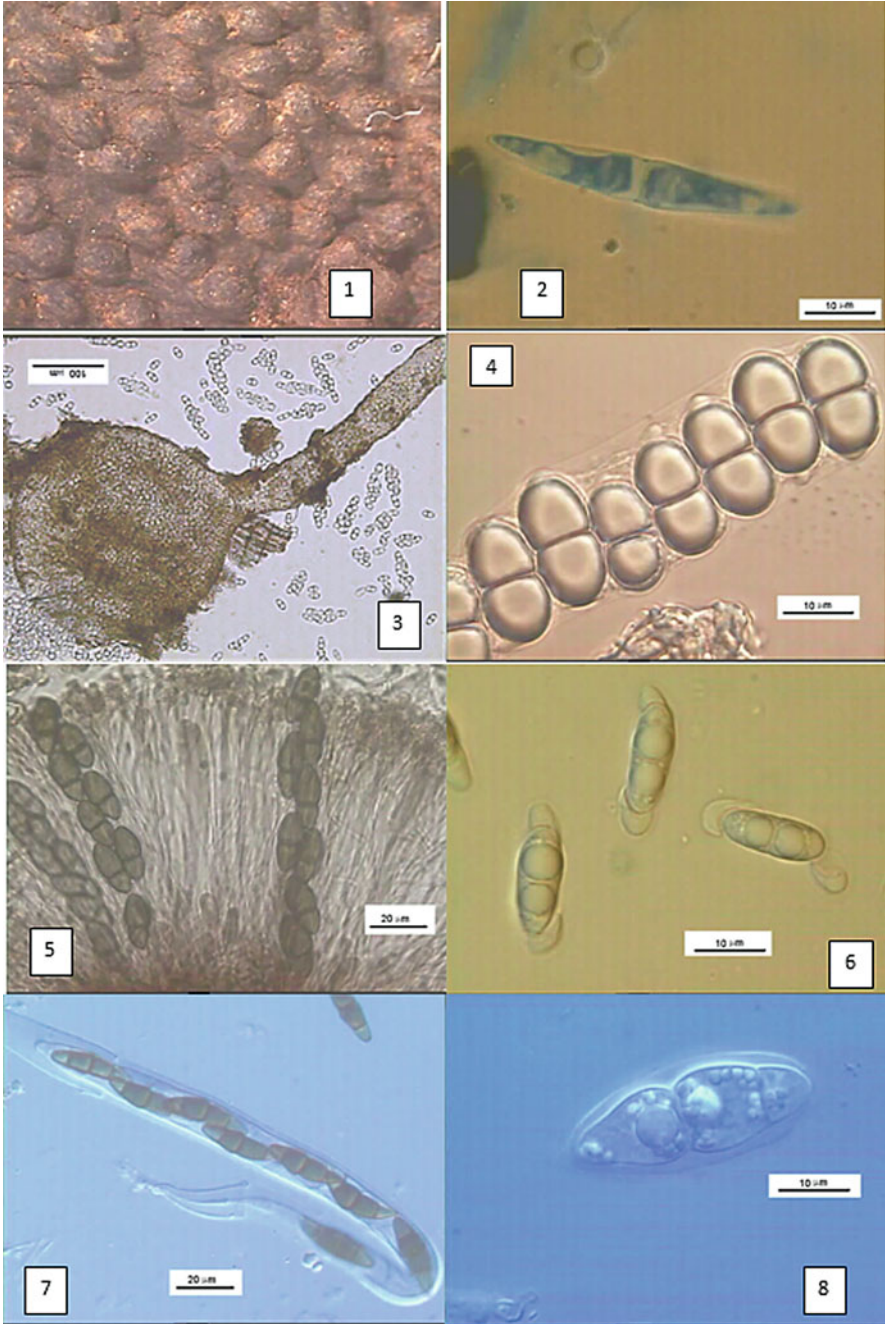

Fig. 9.1 (1) Ascomata of *Rimora mangrovei*. (2) Ascospores of *Rimora mangrovei*. (3) Ascoma of *Tirispora mandoviana*. (4) Ascospores within an ascus of *Tirispora mandoviana*. (5) Ascospores in asci of *Dactylospora haliotrepha*. (6) Ascospores of *Halosarpheia minuta* with apical appendages becoming tapering. (7) *Leptosphaeria oraemaris* ascus with fissitunicate dehiscence. (8) Ascospores of *Morosphaeria ramunculicola*

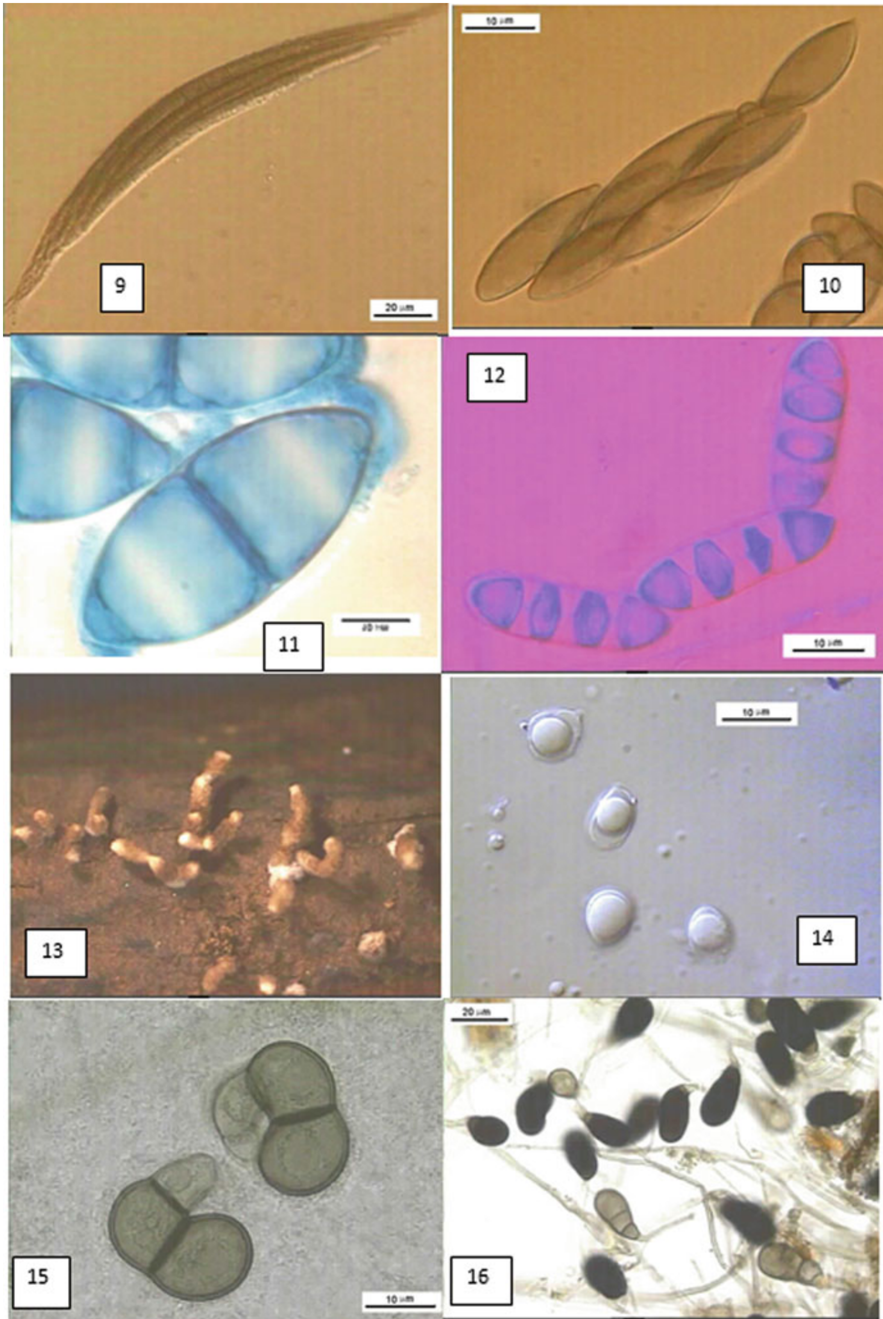

Fig. 9.2 (9) Ascus of *Pedumispora rhizophorae*. (10) Ascospores of *Rhizophila marina*. (11) Ascospores of *Saagaromyces ratnagiriensis*. (12) *Difrolomyces marinospora*. (13) Basidiomata of *Calathella mangrovei*. (14) Basidiospores of *Calathella mangrovei*. (15) Conidia of *Cirrenalia basiminuta*. (16) Conidia of *Trichocladium achrasporum*

having persistent asci with a discoidal apical ring and often an active dispersal of ascospores (Jones 1995; Hyde et al. 1998) and the other group restricted to marine environment which has taxa with early deliquescent asci releasing ascospores into ascomatal centrum and the ascospores ornamented with elaborate appendages or mucilaginous sheaths (Jones 1994, 1995). The marine non-halosphaeriaceous unitunicate ascomycetes are either restricted to marine habitats, e.g., *Marinosphaera*, *Rhizophila*, and *Swampomyces*, or have genera often found in terrestrial, freshwater, and marine environments, e.g., *Anthostomella*, *Linocarpon*, and *Phomatospora*. The marine fungi belonging to *Dothideomycetes* are represented by terrestrial, freshwater, and marine genera, and these occur mostly in intertidal habitats/mangroves. They have few morphological adaptations other than their usual characteristics of persistent fissitunicate asci and ascospores mostly septate (both phragmosporous and muriform) and in many taxa surrounded by mucilaginous sheaths. According to Jones et al. (2015), "classification of many marine fungi remains a confused and an unresolved issue and is particularly acute for the *Ascomycota*" as is well demonstrated by 70 genera that have been placed under incertae sedis. Concerted efforts on research of marine fungal diversity on *Juncus roemerianus* (Kohlmeyer and Volkmann-Kohlmeyer 2001) and *Nypa fruticans* (Hyde and Alias 2000) have shown that these two hosts are hyperdiverse. Basidiomycetes have been represented by a very few fungi (Figs. 9.40 and 9.41), while many anamorphic fungi have been grouped under *Ascomycetes* based on their teleomorph and anamorph connections in cultures or based on molecular inputs (Figs. 9.42, 9.43, 9.44 and 9.45).

Jones (2011) has listed the following habitats/substrates as not fully explored: (1) unidentified species on a range of substrata; (2) marine-derived fungi isolated from sediments, sand, and water; (3) planktonic fungi; (4) deep-sea fungi; (5) non-culturable fungi; and (6) cryptic species.

Marine-derived fungi are considered to be of marine origin but are terrestrial/ freshwater taxa, also called as facultative marine fungi, immigrated from the terrestrial environment. Those fungi that are repeatedly cultured from the marine environment, e.g., species belonging to *Aspergillus*, *Penicillium*, and *Trichoderma* (Overy et al. 2014), are considered as marine, while those that appear only once in the literature are rejected as marine (Jones et al. 2015). The "marine-derived fungi" are mostly isolated from marine animals, macroalgae, mangrove plants, and sediments (Ebel 2012; Debbab et al. 2010).

9.4 Molecular Identification of Marine Fungi

Molecular identification of fungal taxa nowadays is based on the marker gene sequencing. It involves choosing a suitable genetic region which could be easily extracted and amplified that would later on allow sequencing with small amount of DNA/tissue (Reich and Labes 2017). Several marker genes exist having differences in length, resolution, phylogenetic strength, and the number of sequences available in the public domain (Aguileta et al. 2008; Schoch et al. 2012; Kuhnert et al. 2014; Panzer et al. 2015) (Table 9.2). The marker genes could be broadly divided into two

Table 9.2 Loci and primers used in molecular identification of marine fungi (Suetrong et al. 2009; Kuhnert et al. 2014; Devadatha et al. 2018a, b, c)

Locus	Primer name	Oligonucleotides	References
Internal transcribed spacer regions of rDNA (ITS)	ITS1	TCCGTAGGTGAACCTGCGG	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
28S nuclear large subunit rDNA (nLSU)	LROR	GTAGTCATATGCTTGTCTC	Vilgalys and Hester (1990)
	LR5	CTTCCGTCAATTCCTTTAAG	Vilgalys and Hester (1990)
18S nuclear small subunit rDNA (SSU)	NS1	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
	NS4	GTAGTCATATGCTTGTCTC	White et al. (1990)
Second-largest subunit of RNA polymerase (RPb2)	fRPB2-5f	GAYGAYMGWGATCAYTTYGG	Liu et al. (1999)
	fRPB2-7cr	CCCATRGCTTGYTTRCCCAT	Liu et al. (1999)
Translation elongation factor 1-alpha gene (Tef-1α)	EF1-983F	ATGACACCRACRGCRACRGTYTG	Rehner (2001)
	EF1-228R	GCYCCYGGHCAYCGTGAYTTYAT	Rehner (2001)
Beta-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Glass and Donaldson (1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson (1995)

types, namely, (1) sequences belonging to nuclear ribosomal DNA (nuc rDNA) and (2) protein-coding regions. Between these two, the sequences amplified through PCR belonging to nuclear rDNA cluster seem to be more trustworthy than the protein-coding genes (Schoch et al. 2012). Further, the existence of tandem repeats in the nuc rDNA cluster of the fungal genomes makes the sequences easily extractable and amplifiable (see Reich and Labes 2017).

9.5 DNA Barcoding

Rapid and precise identification of fungi is essential to (1) conserve and sustainably use biodiversity, (2) have better ecological monitoring, (3) prevent and control fungal pathogens, (4) quarantine and control exotic species, and (5) be used in human health. Barcoding of sequences of fungi may help to identify cryptic species

within the existing fungal species in addition to helping the biodiversity studies (Xu 2016). The internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster have been recommended to be used as primary fungal barcode by the International Fungal Barcoding Consortium (Schoch et al. 2012). One of the advantages of employing ITS as a barcoding region is that the haploid genome, which usually comprises multiple tandemly repeated copies of the ribosomal rRNA gene cluster, including ITS, makes it possible to amplify this gene from small portions of biological materials (Xu 2016). However, in many cases, single gene locus may not be sufficient for identification, and it may require concordance of multiple gene genealogies through phylogenetic approaches (Taylor et al. 2000), which may serve as secondary and tertiary barcode loci, for proper identification (Xu 2016). Some of the commonly used primers other than ITS are listed in Table 9.2. Many other markers, including novel markers, are also used but are not listed here.

9.6 Internal Transcribed Spacer Region

Among the nuclear ribosomal DNA clusters, the ITS region, united at the time of posttranscriptional processing of the nucrRNA cistron, contains two fragments in eukaryotes. While ITS1 is positioned in between the 18S and 5.8S rRNA gene sequence, the ITS2 is positioned amidst the 5.8S and the 28S rRNA gene sequence (Reich and Labes 2017). Both regions together comprise 400–600 bp in length and occasionally 1000 bp as is the case in many *Dothideomycetes* taxa (Seifert 2009). It is well known that ITS has been recommended as the main DNA barcode of fungi due to the reasons that the variability between species to species is higher than within a species in several groups of fungi besides the fact that PCR success rate is more (Schoch et al. 2012). But then, it is found in many fungi, e.g., basal fungal lineages, the differences in ITS are often not enough for definite species identifications, and this aspect is important for marine fungi since many of them predominantly belong to basal fungal lineages (Panzer et al. 2015; Gutierrez et al. 2016; Taylor and Cunliffe 2016) that can predominate assemblages on the surface of seawater (Richards et al. 2015, Reich and Labes 2017).

9.7 LSU (28S rRNA Gene Sequences)

The molecular sequence length of this marker gene is 2900 bp and has a DNA barcoding capacity at the generic level employing a barcode span of 400 bp (Liu et al. 2012). This marker has a higher possibility of right classification for basal fungal ancestries as well as *Basidiomycota* (Schoch et al. 2012). It is used as a standard marker for the identification of basidiomycete yeasts (Fell et al. 2000; Kurtzman et al. 2011). This marker has been less used in marine fungal research, and the number of sequences available in public domain is very low (Panzer et al. 2015).

9.8 SSU (18S rRNA Gene Sequences)

The small subunit or 18S rRNA gene sequence has a length of 1900 bp and is used for phylogenetic analysis as well as DNA barcoding in some groups of fungi. It has more accuracy in resolving at species level for *Saccharomycotina*, in addition to basal fungal ancestries, unlike the other usually employed fungal barcodes (Schoch et al. 2012), and is the predominant gene marker in public databases for marine fungi (Panzer et al. 2015). This marker gene is a strong one in phylogenetic analyses and is used to recognize yeasts as the predominant morphological group in the deep seas (Bass et al. 2007) besides being used to discover marine fungal diversity at large (Richards et al. 2012, Panzer et al. 2015, Reich and Labes 2017).

9.9 Protein-Coding Sequences

Among the protein-coding genes, only RPB1 (gene region coding largest subunit of RNA polymerase II) and RPB2 (gene region coding second largest subunit of the RNA polymerase II) genes are used for DNA barcoding. One of the advantages of using protein-coding genes in phylogenetic analyses is the prospect of also employing the related amino acid sequences as backbone to correct the errors in sequencing. While RPB1 separates almost all morphospecies of *Glomeromycotina* properly (Stockinger et al. 2014), it exhibits a low fidelity on basal fungal lineages (Schoch et al. 2012) to which many marine fungi belong. Although it has some limitations, this marker is nowadays used as a standard in multi-loci phylogenetic studies (Lutzoni et al. 2004; James et al. 2013, Reich and Labes 2017). RPB2 has been advocated to be used as an alternative fungal DNA barcode (Vetrovsky et al. 2016). Among other protein-coding markers, tubulin is a protein which is an important component of microtubules and comprises α- and β-chains. The tub-2 gene codes β-tubulin chain and is the third widely used marker in fungal multi-loci phylogenies (Feau et al. 2011). One of the drawbacks of the tub-2 gene sequence has been the prevalence of paralogues that often could be misconstrued as tub-2 gene sequences (Hubka and Kolarik 2012). Yet another protein-coding marker is tef1, coding for the translation elongation factor 1 (EF1)-α that has a chain length of 400 amino acids. This marker also helps in resolving the evolutionary relationships of fungi at higher hierarchy (Kristensen et al. 2005). Few studies have undertaken employment of a combination of these protein-coding genes along with nuclear DNA genes for phylogenetic analyses of marine fungi (Devadatha et al. 2018a, b, c).

9.10 Omics and Marine Fungi

Next-generation sequencing (NGS) techniques have opened a new avenue for marine fungal biodiversity research which unfolded an incredulous fungal diversity and richness in the marine realm (Amend et al. 2012, Richards et al. 2015, Rämä

et al. 2016). One of the advantages of this technique is that it deciphers interactions and factors influencing dynamics of fungal communities (Taylor and Cunliffe 2016; Reich et al. 2017).

Metabarcoding banks on amplification of comparatively short marker gene regions often result in a bias due to inadequate resolution of taxa and PCR errors. This is circumvented to some extent by multi-gene phylogenetic analyses as they improve resolving strength, though still PCR dependent. These limitations of metabarcoding can be overcome by third-generation sequencing technologies that allow single-molecule template-based sequencing and generation of read lengths up to many kilobases (Schadt et al. 2010). These single-molecule sequencing (SMS) technologies eradicate a number of PCR-induced errors that often prop up during PCR amplification. They mimic metagenomics approach, where a number of discrete genomes of a sample are fragmented and sequenced. In the context of marine fungi, which represent a minor part of micro-eukaryotic community, exhaustive sequencing is apparently required to get enough extent of reads for a reliable classification (Reich and Labes 2017). On the contrary, the identification of individual taxa, in the future, would most probably be based on the sequencing of the whole genomes, which provide a higher resolution to phylogenetically assign different fungal lineages into a phylogenetic perspective (Wang et al. 2009) in addition to simultaneously providing complete genetic background information on gene clusters that code for secondary metabolites. These two approaches, however, highly rely on a robust downstream bioinformatic analyses for which new standards and bioinformatic tools need to be developed for easy fungal identification. Invariably, such an initiative requires an international, integrated, and multidisciplinary approach for research on marine fungi (Reich and Labes 2017).

The NGS methods are widely used for detecting multiple genes of interest (Culligan et al. 2014), and the advantage of genome-based data generation, nowadays, has been widely used for many fungi (Grigoriev et al. 2014). However, the genome and genetic data on marine fungi and particularly the secondary metabolite-producing gene clusters are less represented as of now in the databases (Reich and Labes 2017).

Proteomics can reflect on fungal responses to the environment to provide complementing information on variations in genomes and transcriptomes. Since proteomics is not affected by posttranslational and transcriptional modifications or phosphorylation events, it can provide a genuine measure of a phenotype (Reich and Labes 2017). It helps in understanding the adaptations and colonization processes of fungi, which have quite similar genomic elements (de Vries et al. 2017), though have different life history patterns.

An investigation on culturable mycocommunities of a deep-sea sediment core sampled from the Canterbury Basin of New Zealand revealed 200 filamentous fungi (68%) and yeasts (32%) belonging to *Ascomycota* and *Basidiomycota*, suggesting the survival, adaptation, growth, and interaction of these fungi with other microbial communities, thus highlighting that the deep-sediment habitat is another ecological niche for fungi (Redou et al. 2015).

9.11 Diversity of Non-culturable Marine Fungi

The culture-independent analyses of fungal diversity in the sea led to the discovery of novel lineages of fungi such as marine *Chytridiomycota* lineage in deep-sea sediments (Nagano et al. 2010), unknown phylotypes diverging within the basidiomycete radiation in deep-sea hydrothermal vents (Le Calvez et al. 2009), and 36 novel marine lineages (Richards et al. 2012).

Fungi belonging to *Malassezia* (*Malasseziomycetes*) were retrieved from deep-sea sediment samples (Lai et al. 2007) as well as hydrothermal vents (Le Calvez et al. 2009). This genus is otherwise known predominantly from both healthy and diseased human skin and hence Amend (2014) raised the question whether it is truly marine, even though it has been reported in a great diversity of habitats such as polar regions and deep-sea vents.

Some workers who studied culture-independent mycota from marine habitats have added environmental fungal sequences that branch out from the known fungal taxa (Bass et al. 2007; Gao et al. 2008, 2010; Jebaraj et al. 2010; Singh et al. 2011, 2012; Arfi et al. 2012; Thaler et al. 2012). Several studies have shown active roles of fungi in the marine habitats that are involved in the biogeochemical cycling (Alexander et al. 2009; Edgcomb et al. 2011). Marine environments can be basically divided into coastal and deep-sea regions. The studies on the molecular diversity of fungi from coastal realms are very few in contrast to deep-sea regions (Manohar and Raghukumar 2013).

9.12 Coastal Habitats

Among the coastal regions, the coasts of Brazil (Cury et al. 2011), coral reeves of Hawaii (Gao et al. 2008, 2010), and mangrove forests (Arfi et al. 2012) were studied, to mention a few, on molecular studies of fungi, and they report sequences belonging to *Ascomycota*, *Basidiomycota*, and *Chytridiomycota* in addition to certain novel environmental clusters. A rich diversity in environmental sequences from coral reefs has been identified (Gao et al. 2010; Singh et al. 2012). Mangrove habitats are also rich in marine fungi, and the direct examination method shows the dominance of *Ascomycetes* (Hyde and Sarma 2000); many marker gene sequence-based studies also have been carried out to this effect (Hyde et al. 2011). However, an investigation (Arfi et al. 2012) based on pyrosequencing approach in mangroves has shown that *Agaricomycetes* are the predominant group.

9.13 Deep-Sea Environment

According to Manohar and Raghukumar (2013), the deep-sea environment is characterized by lower temperatures, higher hydrostatic pressures, lack of light, and a very low biological diversity.

Studies by Damare et al. (2008) and Singh et al. (2010) showed that the species diversity is low in deep-sea sediments as compared to coastal sediments and common terrestrial forms. Many different types of micro-eukaryotes such as novel marine alveolates, dinoflagellates, and stramenopiles were reported from deep-sea regions (López-Garcia et al. 2007; Edgcomb et al. 2002) in addition to novel fungal phylotypes using universal micro-eukaryotic primers. Fungal-specific metagenomic analyses also revealed hitherto unknown species richness (Le Calvez et al. 2009). Since basal groups of fungi were not represented in studies on vent regions, in the future, usage of specific primer sets for each phylum should be resorted to, in order to better characterize fungal distribution (Manohar and Raghukumar 2013). RNA-based libraries may also help in recovering novel sequences in addition to providing clues on metabolically active sequences that play a part in the deep-sea ecosystem (Edgcomb et al. 2011). Since the initial fungal branching is known to have occurred in a marine domain, future molecular studies along the lines mentioned above may provide an understanding on the fungal evolution (Manohar and Raghukumar 2013).

The main groups that are found in marine habitats are *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and basal fungal clades. Within *Ascomycota* three environmental groups have been recognized, namely, (1) deep-sea fungal group (DSF group I) with major cluster identified being *Saccharomycotina* that clades around *Candida* sp. (Nagano et al. 2010; Thaler et al. 2012); (2) *Pezizomycotina* clonal group (PCG) that branches close to common fungal cultures belonging to the genera *Eupenicillium*, *Penicillium*, and *Aspergillus* (Lopez-Garcıa et al. 2003; Dawson and Pace 2002); and (3) soil clone group (SCGI) that clusters between *Taphrinomycotina* and *Saccharomycotina* as a monophyletic clade (Nagahama et al. 2011). In the case of *Basidiomycota*, *Ustilaginomycotina* new environmental sequences have clustered together into hydrothermal and/or anaerobic fungal group (Hy-An group) (López-Garcia et al. 2007). Since the earliest work on this group came from anoxic vent habitat, such a name has been given. However, it has been shown that it has representative sequences from all other marine habitats such as coastal, deep-sea, and oxygen-depleted environments. More number of novel environmental sequences were recorded for *Chytridiomycota* and basal fungal lineages with almost half chytrid population belonging to *Cryptomycota* (Jones et al. 2011). The sequences cluster close to the chytrid genus *Rozella* (Bass et al. 2007; Nagahama et al. 2011). While only a few fungal sequences belonging to *Zygomycota* were represented, other phyla such as *Glomeromycota*, *Blastocladiomycota*, and *Neocallimastigomycota* were not represented in any of the molecular studies (Manohar and Raghukumar 2013).

Relatively few studies have been conducted on environmental fungal sequences from coastal regions (Arfi et al. 2012; Cury et al. 2011; Gao et al. 2008, 2010; and Wegley et al. 2007) or hydrothermal vents (Edgcomb et al. 2002, 2011; Le Calvez et al. 2009; Lopez-Garcıa et al. 2003, 2007) when compared to deep-sea regions (Bass et al. 2007, Lai et al. 2007; Lopez-Garcia et al. 2001; Nagahama et al. 2011; Nagano et al. 2010; Singh et al. 2011, 2012; Thaler et al. 2012).

More molecular data for each taxonomic group need to be made available, and a diligent phylogenetic analysis needs to be carried out to unravel the yet to be described marine fungal diversity (Manohar and Raghukumar 2013).

Polymerase chain reaction (PCR)-based studies on eukaryotic small subunit ribosomal RNA genes revealed a low recovery of fungal sequences from the surface marine water columns of coastal (Richards and Bass 2005) and open-water environments (Massana and Pedrós-Alió 2008). The meta-analyses of marine water column samples involving 1349 clones belonging to 23 coastal libraries and 826 clones belonging to 12 open-water libraries resulted in the recovery of only 16 fungal clones (0.8%), which is suggestive of a lower diversity and abundance of fungi in open-water columns, or it could be that the methodologies employed were prejudiced against recovery of fungal sequences (Massana and Pedrós-Alió 2008). This is in contrary to freshwater analyses where a rich diversity and relative abundance of fungal OTUs have been demonstrated (Lefranc et al. 2005; Lefèvre et al. 2007; Jones et al. 2011). Meta-analysis of clone library sampling of marine fungi demonstrated dominance by Dikarya followed by sequences that branch with chytrids (Hassett and Gradinger 2016, Richards et al. 2012).

The abundance and diversity of fungal sequences also seem to be influenced by the techniques followed. While surface marine water column studies have shown a low diversity and abundance, the clone library analyses using DNA retrieved from deep-sea environments have resulted in a higher correlative representation of fungal sequences (Takishita et al. 2006; Edgcomb et al. 2011; López-Garcia et al. 2007; Richards et al. 2015). Studies involving second-generation SSU V4 rR/DNA diversity tag sequencing and meta-transcriptome sequencing indicate that fungi predominate eukaryotic assemblages in deep-sea sediments (Orsi et al. 2013a, b).

A study involving BioMarks V4 SSU Rr/DNA-derived Roche/454 sequence tag dataset (Massana et al. 2015) from 130 samples collected from 6 European marine sites along with fluorescence microscopy to detect eukaryotic microbes that have chitin cell walls was analyzed for diversity and abundance of fungi in water column and surface sediment samples (Richards et al. 2015). They found around 71 fungal "operational taxonomic units (OTU) clusters" that cover 66% of all the sequences assigned to the fungi whose phylogenetic studies revealed a significant number of chytrid-like lineages that were not described earlier, suggesting that marine environment has a rich diversity of zoosporic fungi that are new to taxonomic inventories. Based on this study, i.e., a comparison of relative abundance of RNA-derived fungal tag sequences and chitin cell wall content, they concluded that fungi comprise a low fraction of the eukaryotic communities in the water column samples (Richards et al. 2015).

9.14 Role in the Habitat/Environment

Marine fungi mainly serve as saprobes involved in the nutrient-regeneration cycles (Hyde and Lee 1995; Hyde et al. 1998; Jones 2000). Fungi generally have chitin-rich cell walls and obtain nutrients exclusively by feeding osmotrophically, a process that

involves the secretion of depolymerizing enzymes followed by the transportation of nutrients, usually as digested monomers, back into the cell (Richards et al. 2012). As a result of this lifestyle, many fungi seem to have lost the ability to perform phagocytosis and hence cannot engulf and digest prey cells in the same way as other eukaryotes could do. Accordingly, this reliance on osmotrophy determines their survival in ecosystems, as they thrive in nutritionally rich environments including plant and animal hosts, soils, sediments, and detritus environments, where they can adhere to substrata; secrete enzymes; break down complex polymers such as carbohydrates, proteins, and lipids; and take up nutrients (Richards et al. 2012). This combination of characteristics partly may explain why fungi are considered as non-diverse as well as having low abundance in many upper and surface marine water column samples (Richards and Bass 2005).

There are very few fungal pathogens that attack coastal plants that have been reported. Some reports are suggestive of Vesicular Arbuscular Mycorrhizal (VAM) fungal infestation in mangrove habitats (Sengupta and Chaudhuri 2002; D'Souza and Rodrigues 2013). However, not many studies have been conducted on mycorrhizae from coastal environments. Earlier there were not many reports on lichenized fungi from coastal habitats. However, recently, several lichenized fungi have been reported. The symbiotic association between algal component and fungal component may make the lichen survival much better, and most of them are found in the aerial parts of the hosts. Many earlier workers were interested only in marine fungi which occur in the woody materials or dead plant vegetation immersed in marine waters. Hence they neglected the lichen diversity. Moreover, we need experts to study the lichenized fungi from coastal habitats particularly the mangroves. Though endophytic fungi colonizing living leaves in coastal habitats are not considered as marine fungi, the fact that the plants in the marine habitats have to excrete salts would make the fungi a harsh environment to survive, which in turn should make them become halotolerant. A number of studies on endophytic fungi in mangroves have been reported both on diversity (Suryanarayanan et al. 1998; Kumaresan and Suryanarayanan 2002) and the metabolites (Suryanarayanan et al. 2009; Deshmukh et al. 2018). The typical marine fungi (obligate marine fungi) are slow growers and difficult to maintain. Apparently only a few attempts have been made to screen them, and some bioactive compounds have been reported from these studies (Bugni and Ireland 2004). If these obligate marine fungal cultures could be scaled up to the extent that sufficient extracts for isolation of the novel compounds and their characterization are available, then the compounds could be mimicked to produce through synthetic chemistry route. In the case of the formerly called "marine-derived fungi," they are already fast growing, and hence their production of compounds in fermenters should not be a problem. More than 1000 compounds have been reported from fungi isolated from marine habitats (Deshmukh et al. 2018).

Marine fungi produce a battery of enzymes. Due to the continuous agitation of marine waters, there would be less scope for production of macroscopic fruit bodies belonging to basidiomycetes on drift wood or any other plant substrata. Hence, the *Ascomycota* dominate on these substrates. These marine fungi also have been shown to have adopted in such a way that they could produce lignin-degrading enzymes

which have been usually a domain of basidiomycetes (Raghukumar et al. 1994; Rohrmann and Molitoris 1992; Pointing 1999; Bucher et al. 2004). In the applications side, a significant decolorization of textile effluents and synthetic dyes by marine-derived fungi has been reported by Raghukumar et al. (1996, 2004, 2008) and D'Souza et al. (2006). These are the studies, to mention a few, available on biotechnological applications of marine fungi. However, there is a huge potential to be realized in the future.

9.15 Potential Applications

Marine fungi are a rich source of bioactive compounds, including several novel compounds that have antibacterial, anticancer, anti-inflammatory, antiplasmodial, and antiviral properties (Bugni and Ireland 2004; Bhadury et al. 2006; Saleem et al. 2007; Blunt et al. 2016). Most of the metabolites reported so far are from asexual states of ascomycetes, e.g., *Penicillium* and *Aspergillus* spp., the basidiomycetes, also show metabolites of interest (Imhoff 2016). In the area of polycyclic aromatic hydrocarbon degradation, Passarini et al. (2011) reported a 99.7% pyrene and 76.6% benzopyrene degradation by *Aspergillus sclerotiorum* after 8 and 16 days, respectively. Raghukumar et al. (2006) have shown that two non-identified isolates of marine-derived fungi were able to eliminate phenanthrene from culture media by adsorption onto fungal mycelium. In another study, *Aspergillus* sp., isolated from marine sediment from China coast, showed the ability to degrade benzo(a)pyrene up to 30% in 3 days and 60% in 12 days (Wu et al. 2009). Raghukumar et al. (2004) demonstrated efficient lignin mineralization by a basidiomycete retrieved from a decaying sea grass. Once again, these are few examples available, and a lot more need to be done in this area in exploring more fungi.

9.16 Future Perspectives

There are large tracts of mangroves in Africa and Asia, including Indonesia, Sundarbans of India, etc., which are yet to be fully investigated for the marine fungi. The taxonomists are becoming an endangered species and hence future marine mycologists need to be trained for the continuity.

The culture-independent investigations on marine fungal diversity are of recent origin, hence requiring a greater sampling to get an insight on their occurrence as well as distribution (Pang and Jones 2017).

There is a discussion going on in the scientific circles as to whether DNA sequence data could be taken as types of fungal taxa or not. According to Lucking and Hawksworth (2018), using specimen or culture-based inventories alone, the gap between known and estimated fungi cannot be filled. However, sequences with unidentified fungi may help in bridging this gap. One of the serious drawbacks of sequence-based nomenclature is the prospect of parallel classifications, either by describing taxa which already have names based on physical types or by usage of

different markers to delimit species within the same lineage. However, the predictable error seems to range between 1.5% and 20.4% when compared to 30% in physical types. To circumvent any duplication in classifications dependent on diverse gene regions, Lucking and Hawksworth (2018) suggested that sequence-based nomenclature (SBN) has to be confined to a single marker, and among different markers, fungal ITS barcoding marker is preferable since the aim is more of naming lineages to produce a reference database that is independent of these lineages representing a species, closely aligned species complexes or infraspecies, etc. and not accurate species delimitation. In support of SBN, they further argue that clustering methods are inappropriate and instead phylogenetic methods based on multiple alignments, combined with quantitative species recognition methods are better. Besides providing strategies to obtain higher-level phylogenies, they concluded by saying that "voucherless SBN is not a threat to specimen and culture-based fungal taxonomy but a complementary approach capable of substantially closing the gap between known and predicted fungal diversity." On the other hand, Thines et al. (2018) listed ten reasons why an SBN is not useful for fungi any time soon. The ten reasons are (1) the resolution of barcoding loci, especially ITS, varies among different groups; (2) there is a high risk of introducing artifacts as new species; (3) there is no consensus regarding the data type or amount needed for species delimitation; (4) voucherless data are not reproducible; (5) sequence-based types cannot be verified; (6) sequence-based types are not relatable; (7) sequences of reported OTUs are derived, not actual sequences; (8) sequence-based types favor well-funded large mycology labs and leave researchers in developing countries behind; (9) allowing sequence-based types would be detrimental for mycology as a discipline; and (10) an introduction of sequence-based nomenclature is impossible at present because of the rapid progress with which sequencing technologies are developing it would be difficult to predict which type of sequencing technique to be adopted and the length of the gene or genome to be considered for comparisons. These reservations expressed by Thines et al. (2018) seem to be realistic for the present, but in the future, the SBN may be acceptable once the reasons cited are addressed. Hence, in the future, it may also be helpful to marine mycologists to study the fungal diversity in marine environments, once SBN approach is accepted by the mycological fraternity. It should be remembered, however, that the reservations about SBN approach are only about considering environmental sequences as "types" of fungi and not otherwise.

As of now, we have techniques that allow propping up of only the fast-growing fungi in culture media. However, the recent methods adopted by Arnold et al. (2007) and Collado et al. (2007) allow isolation of even slow-growing fungi. Such techniques should be widely used in marine fungal research so that instead of the routine isolation of fast-growing fungi, the rare fungi could also be isolated and that the same may provide a true picture about the diversity of fungi in marine environments. Further techniques that would allow identification of individual fungal species from environmental sequences, in the future, would be more worthwhile.

9.17 Conclusions

Richards et al. (2012) mentioned that fungi seem to be rare in marine habitats since there are comparatively few marine isolates in cultures, and culture-independent sequence studies on eukaryotic microbial diversity from environmental DNA samples based on small subunit DNA (SSU rDNA) indicate rare recovery of marine fungal sequences from clone library experiments. However, Pang and Jones (2017) opined that while interpreting the results of molecular studies, caution should be taken because seawater is a highly diluted and spreading medium and not a growth substrate, and accordingly, fungi in these habitats may not denote the active marine populations as many fungal propagules could be of freshwater/terrestrial origin in contrast to sediments which represent a niche for amassing of fungal propagules. Commenting on low fungal abundance found by Richards et al. (2015), who used RNA tag sequencing and fluorescent staining of samples, Pang and Jones (2017) mentioned that seawater does not support a steady population of fungi because it contains low organic matter with exception being phytoplankton. Many recent molecular studies revealed the predominance of *Ascomycota* and *Basidiomycota* in seawater/sediments (Jones et al. 2015; Richards et al. 2015; Picard 2017; Hassett et al. 2017), however without acknowledging individual species which made comparisons more difficult (Pang and Jones 2017). The wide gaps in the three approaches of marine fungal research, namely, (1) substrate-based studies, (2) isolations from seawater, and (3) sampling of DNA from water and sediments, many times from greater depths in the ocean, are not appreciative (Pang and Jones 2017).

Reich and Labes (2017) opined that one of the drawbacks of marine fungal researches is that the culture-dependent and culture-independent approaches are often undertaken separately, and the available datasets are not interwoven. Accordingly, they felt that the two groups of (1) natural product researchers and (2) biodiversity researchers and ecologists, presently two divergent scientific communities, having their own languages and priorities, should be united.

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Diversity of Iron and Sulphur Oxidizers in Sulphide Mine Leachates

10

S. R. Dave and D. R. Tipre

Abstract

Mining of metals and fuel is inevitable, as we need both these minerals for development. Mining process exposes a large surface area of minerals to air and water, which enhances chemical oxidation of ferrous and reduced sulphur compounds that results in generation of protons. *Thiobacillus* and *Thiomonas* initiate their activity at neutral pH, leading further acidification of environment and pH reduction to 5.0. At this stage, acidophilic sulphur and iron-oxidizing organisms accelerate the process, and pH falls below 3.0 and many a time, it reaches even lower than 1.0. Acidophilic iron and reduced sulphur-oxidizing organisms are versatile and found in all the three microbial domains. They use inorganic or organic substrates as electron donor; some of them use both iron and sulphur as a source of energy. In terms of optimum temperature for growth, they are mesophilic, thermophilic, extremely thermophilic and psychrotolerant. Biooxidation of iron and reduction of sulphur compounds form acid mine drainage and pollute several thousands of miles of water streams globally. The formed acid mine drainage dissolves several metals and other materials resulting in the formation of metal-loaded acidic turbid polluted waters. If the organisms responsible for acid mine drainage are used scientifically, they are helpful for metal extractions from mining waste, ores, concentrates, e-waste and municipal solid waste. The activity of iron-oxidizing organisms is also used for bioremediation of iron from acidic water and production of yellow/brown pigments. Till now diversity of a few microbes is known; therefore, further work is needed to explore them for biotechnological applications.

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Archaea · Bacteria · Acid mine drainage · Bioleaching · Microbial diversity · Iron and sulphur oxidizers

10.1 Introduction

The rapidly growing uncontrolled urbanization and industrialization need more and more minerals to fulfil the requirements of metals and fuels. Raising demand for these minerals has forced extensive open-cast or underground mining. Excavations of minerals from the Earth's crust to the Earth's surface, or even blast at mine, bring harmful chemicals along with minerals to the surface of the Earth and increase their contact with water and air (Kaur et al. 2018). Thus, all the mining activities result in the generation of one or other types of pollution. The emissions of toxic gases at mining sites pollute air, and seepages of contaminated mine water pollute water and land. The generation of acidic water as drainage from the active and abandoned mines, tailing dump and mine overburden, is known as acid mine drainage (AMD) or acid mine leaching (Méndez-García et al. 2015). AMD is defined as metal-rich acidic water formed due to the chemical or biological reactions between waste ore, mine overburden, tailing or even minerals themselves with water, air and microbes. Mining activities and drainage of contaminated mine water result in the formation of an extreme environment; it is one of the major problems worldwide due to its extremely low pH, high salinity and elevated metal and sulphate concentration (Kamika and Mombu 2014; Adler et al. 2007; Auld et al. 2013; Sajjad et al. 2016). AMD is responsible for contaminating several thousands of miles of water streams globally. One such long stretch of >8000 km in the eastern United States is reported to be polluted due to heavy metal-loaded acidic drainage from an abandoned coal mine (Macalady et al. 2013). Similar pollution problems due to acid mine drainage are reported in many counties throughout the world (Bosecker et al. 2004; Breuker et al. 2009; Kamika and Momba 2014; Dave and Tipre 2012). AMD has a significant adverse effect on the flora and fauna in the near vicinity of the mines and even several hundred miles from the mined site (Kaur et al. 2018). Acid drainage also takes place at non-mining areas such as construction sites or naturally in some environments, and such a process is known as acid rock drainage (ARD). Due to huge volumes of AMD generated at almost all mining sites and the presence of metallic pollutants and dissolved solids, they are responsible for global public health hazards and environmental pollution with social and economic impact (Oelofse 2009). Once AMD is formed, it is very difficult to control or prevent, and it will continue for hundreds of years even after the mine is closed. Roman-era mine is still generating the acidic drainage (Coil et al. 2014). In the first stage, ferrous iron present in the minerals or in mine water gets auto-oxidized when it

comes in contact with air and water at neutral or alkaline pH and forms ferric iron, which gets precipitated due to the low solubility of ferric iron in the neutral water. In the process of hydrolysis, when ferric iron precipitates, it releases some protons that results in lowering of the system pH. At neutral pH, species of neutrophilic sulphur bacterial genera like *Thiobacillus* and *Thiomonas* initiate oxidation of available sulphur or reduced sulphur compounds and bring down the pH to about 4.5, and subsequently, sulphur- and iron-oxidizing acidophiles take over the charge and reduce the pH further (Leduc et al. 2002; Johnson and Hallberg 2003). However, once pH becomes acidic (< 5.0), auto-oxidation of ferrous iron seizes or becomes negligible.

Microorganisms are ubiquitous; they survive and grow in extreme ecosystems too. Microorganisms oxidize sulphur or reduced sulphur compounds at much higher rates as compared to chemical oxidation. The oxidation of iron and reduced sulphur compounds under acidic environments releases considerable energy, which stimulates the growth of diverse carbon dioxide-fixing autotrophic organisms. Autotrophic acidophilic iron- and sulphur-oxidizing organisms are the key players for most of the AMD and in this ecosystem; along with autotrophs several acidophilic heterotrophic organisms also grow (Rawlings and Johnson 2002). It is considered that most of the extreme AMD are created by microbial metabolism of iron and reduced sulphur compounds. However, only in the year 1947, Colmer and Hinkle have isolated acidophilic autotrophic iron- and sulphur-oxidizing bacteria from AMD (Rawling 1997; Dave and Tipre 2011) and provided the first scientific authentic proof on the role of microorganisms in metal solubilization and oxidation of ferrous sulphate and sulphur. AMD represents extreme acidic metal-loaded ecosystems that are considered being simple in terms of types of microorganisms present. Rawlings and Johnson (2002) stated that organisms, which grow optimally at pH 3.0 and below, are considered to be extreme acidophiles; however, the exact demarcation between moderate and extreme acidophily is doubtful and debatable. As per this definition, acidophilic organisms are found to be distributed in all the three biological lineages, Archaea, Bacteria and Eukarya, but not those which are responsible for bringing down the pH of the environment below 3, but unable to grow in the medium of pH 3.0. In extreme AMD environments, the levels of environmental factors create problems for the survival of one or more taxa (Wang et al. 2011; Johnson and Hallberg 2003).

Understanding the microbial diversity of AMD may be useful to know the interactions between autotrophic and heterotrophic iron and sulphur oxidizers and harness their enzymes and metabolic pathways for biotechnological applications such as metal extractions from minerals, mine wastes, e-waste and even municipal solid waste. It is also useful in preventing and controlling AMD generation at active and abandoned sulphidic and coal mines. In this context, attempts are made to illustrate the diversity of iron- and sulphur-oxidizing microorganisms in general and acidophiles in particular and their environments as well as biotechnological potentials.

10.2 Diversity of Culturable and Non-culturable Acidophilic Iron- and Sulphur-Oxidizing Microbes

Biodiversity commonly refers to the variety and variability of life on Earth. Microorganisms are the smallest form of life on planet Earth, and they are omnipresent wherever there is a possibility of life. Among three domains of life, Archaea and Bacteria are represented by prokaryotic microbes and eukaryotes in the third domain. If we look at iron- and sulphur-oxidizing microorganisms, they too are distributed in all three domains of life forms. Many iron- and sulphur-oxidizing organisms are extremophiles, which are difficult to cultivate in the laboratory. Moreover, their generation time is also long as compared to many heterotrophic organisms growing in normal environments. In order to understand their environmental importance and biotechnological utility, polyphasic approach is adopted for studying extremophilic iron- and sulphur-oxidizing organisms.

10.2.1 Approaches for Studying the Diversity of Iron- and Sulphur-Oxidizing Organisms

To study microbial diversity, two approaches are widely used—biochemical and molecular—but none of the approaches allow the complete analysis of microbial diversity. Biochemical approach includes plate count, community physiological profiling (CLPP) and fatty acid methyl ester analysis (FAME). This approach shows certain limitations as in the case of plate counts, less than 10% of organisms are culturable and the remaining are uncultivable, therefore, not detected. Moreover, this approach shows bias towards fast-growing microbes, which will not allow the growth of slow growers or their growth is dominated by the fast growers, thus escaping detection (Tabacchioni et al. 2000). CLPP represents the culturable fraction of the community, also favours fast-growing organisms and represents the available carbon source utilizers. This approach provides information only about potential metabolic diversity, and the method is sensitive to inoculum density (Classen et al. 2003, Garland 1996), while FAME analysis method is influenced by external factors (Zelles 1999). Molecular approach is based on nucleic acids, like G + C content, nucleic acid reassociation and hybridization, DNA microarrays, DNA cloning and sequencing and polymerase chain reaction (PCR)-based methods. The G + C content of taxonomically related groups differs only by 3–5%; though the method is not influenced by PCR biases, it requires a large amount of DNA and does not provide a much finer level of resolution as it depends on cell lysis and DNA extraction efficiency (Tiedje et al. 1999). The DNA reassociation gives a measure of the genetic complexity of the microbial community but requires pure total DNA. It again lacks sensitivity as it depends on cell lysis and nucleic acid extraction; the rate of hybridization depends on the similarity of sequences and may not detect the sequences present in low copy number (Cho and Tiedje 2001). DNA microarrays and DNA hybridization can analyse thousands of genes but can only detect the most abundant species. They need cultured organisms and are found to be accurate in

low-diversity systems (Greene and Voordouw 2003). PCR-based method targets the 16S rDNA for prokaryotic diversity and 18S rDNA and internal transcribed spacer (ITS) region for eukaryotes. The major limitation along with cell lysis and DNA extraction is the choice of universal primers, primer degeneracy and highly variable G + C contents of the amplified DNA molecules, which are known to affect amplification efficiencies (Kirk et al. 2004).

10.2.2 Metagenomic Approach

The development of DNA sequencing techniques directly from environmental samples plays a significant role in understanding the diversity of prokaryotes in a wide variety of ecosystems. Today, metagenome study has become a mandatory tool for studying the microbial diversity and metabolic potential of environmental microbes, whose major portion is not yet culturable. The classical metagenomic approach involves cloning of environmental DNA into vectors with the help of ultra-competent bioengineered host strains. The resulting clone libraries are subsequently screened either for dedicated marker genes that is a sequence-driven method or for metabolic functions, which is a function-driven method (Riesenfeld et al. 2004). The function-driven approach is still widely used for screening enzymes with applied prospects (Ekkers et al. 2012), whereas in microbial ecology, increasing throughput, i.e. base pairs per run, and decreasing costs for DNA sequencing have rendered the sequence-driven methodology largely outdated. Now direct sequencing of environmental DNA is commonly used to study the gene inventories of microbial communities. By linking the resulting metagenomic data with microbial diversity data of 16S rRNA gene amplicon sequencing, in situ expression data (metatranscriptomics and metaproteomics) and environmental parameters, an in-depth complete ecosystem study has become feasible (Simon and Daniel 2011). These comprise considerations about the habitat, sampling strategy, sequencing technology, assembly, gene prediction, taxonomic classification and binning, biodiversity estimation, function predictions and analyses, data integration and subsequent interpretation and data deposition (Teeling and Glockner 2012).

10.2.3 Acidic Environment Habitat of Acidophilic Iron and Sulphur Oxidizers

Acidic environments with pH values of <5 are widely distributed on the Earth, but extremely acidic environments having pH < 3 are not common. Highly acidic environments may be natural or anthropogenic, i.e. man-made extreme acidic environments are mostly related to the biogenic formation and accumulation of sulphuric acid (Johnson and Quatrini 2016; Quatrini and Johnson 2018). Sulphur is one of the most abundant elements in planet Earth. It occurs as pyrite (FeS_2) and other sulphidic minerals in rocks or ore bodies, volcanic areas and geothermal areas. Oxidation of these reduced sulphur compounds generates extremely low pH

ecosystems. The examples of highly acidic ecosystems are Lake Kawah Ijen, Indonesia, having a pH 0.7 and the Copahue volcano, Lake Caviahue area, Argentina, where the pH is 0.2. Yellowstone National Park (Wyoming) is the most in-depth investigated ecosystem and well known for people working on extreme environments (Lohr et al. 2005; Urbieta et al. 2012). Sulphide-rich waters interacting with atmospheric oxygen give origin to sulphuric acid and the emergence of extreme acidic environments (Quatrini and Johnson 2018). Natural extremely acidic environments are linked with the dissolution of sulphidic minerals as they exist but are relatively uncommon as compared to those of anthropogenic origin that have arisen from the mining of metals and coals, which are commonly found in most industrially developed countries (Harrison 2016). The low pH in such ecosystems is mostly due to high concentrations of sulphuric acid, though the pH of mine-impacted environments remain slightly higher than those associated with sulphur oxidation due to the presence of ferrous/ferric iron which buffers the system, for instance, due to sulphidic mineral mining, the water body of Rio Tinto river, Spain, shows ~2.5 pH (Harrison 2016).

Another important abundant reducing component of the planet Earth is ferrous iron. When it is exposed to air, it becomes highly sensitive to oxic-anoxic interfaces where microorganisms transfer electrons from iron to strong oxidants resulting in the suppression of abiotic reactions and it ends up in the formation of AMD (Emerson et al. 2010). Under such acidic condition, iron biooxidation is several orders of magnitude faster as compared to chemical oxidation, which generates more and more ferric iron and soluble metals in the mine drainage, and even under such a low redox potential environment, microorganisms thrive and perform their metabolic activities (Templeton 2011). Due to highly acidic pH, AMD waters are rich in dissolved ferrous and ferric iron, and in some cases, concentration of dissolved ferric iron is many folds higher as compared to dissolved oxygen; thus ferric iron serves as an electron acceptor in microbial metabolism (Backer and Benfield 2003). Apart from the described acidic environments, some of the iron- and sulphur-oxidizing acidophilic organisms are also isolated from soil, sludge, hot water springs, environment contaminated with iron scrap and even from seawater. Several microenvironments and microbial niches are detected in AMD system depending on the prevalence of different pH, ionic strength, type of minerals and temperature, and the community of microorganism will develop with a mixture of organisms, but all such habitats support a restricted number of species.

10.3 Diversity of Acidophilic Prokaryotes

The microbial diversity in AMD environments has a direct relationship with the characteristics of the habitats, i.e. the kind of minerals, organic materials, water content, heavy metals, salinity and pH (Rodrigues et al. 2014). In the environment most with mining activities, high concentrations of sulphur or reduced inorganic sulphur compounds (RISCs) and their chemical and biological reactions cause

generation of sulphuric acid which decreases pH to as low as below 3.0, limiting the colonization of microbial communities and imposing selection pressure on specific microbial populations, such as sulphur and iron oxidizers. Organisms present in such environments are referred to as extreme acidophiles; they are widespread in all the three domains within the tree of life (Johnson and Quatrini 2016). Extremely acidophilic prokaryotes are found in α-Proteobacteria, β-Proteobacteria and γ-Proteobacteria phyla. Some species of the class *Acidithiobacillia*, phylum *Nitrospirae* (particularly, genus *Leptospirillum*) as well as phyla *Aquificae* and *Verrucomicrobia* consist of many Gram-negative acidophiles. Gram-positive extreme acidophiles fall within two phyla, namely, *Actinobacteria* and *Firmicutes*; these are metabolically highly versatile among all the extremophilic prokaryotes. In domain Archaea, both Euryarchaeota and Crenarchaeota phyla include extreme acidophiles and thermo-acidophiles. Extreme acidophiles can also be found in domain Eukarya. The acidophiles are distributed in fungi, algae (red and green), diatoms, amoeba, flagellates and ciliates (Johnson and Quatrini 2016; Quatrini and Johnson 2018; Aguilera et al. 2016). Extreme acidophiles exhibit a wide range of physiological characteristics within the community as well as within species living in the ecosystem. The unique ability found among acidophiles is their ability to tolerate and flourish in an extremely acidic environment due to the strategy to maintain the cell internal pH near to neutral against the highly acidic environmental outside the cell. The strategies used by these microbes for their survival include both active and passive mechanisms to maintain cell integrity, which encompasses proton exclusion, exchange, pumping, consumption and neutralization and cytoplasmic buffering. They also show damage extenuation strategies, i.e. DNA repair; and also, synthesis of acid stable proteins that is very commonly found (Baker-Austin and Dopson 2007). However, the contributions of each of these strategies to increase acid tolerance at the community level are still not much known. Apart from this, many acidophiles show resistance to elevated concentrations of various metals like copper, cobalt, nickel and zinc and metalloids like arsenic. Acidophiles tolerate and survive in the presence of high ionic strength environment, i.e. high osmotic potentials arising due to the manifestation of large amounts of inorganic solutes. These groups of organisms also resist variable temperatures and redox potentials (Quatrini and Johnson 2018). Distribution of acidophilic iron and sulphur oxidizers in terms of their optimum growth temperature is shown in Fig. 10.1. They mainly constitute mesophilic and thermophilic groups; however, some members are reported as psychrotolerant, and some members of Archaea are extreme thermophiles. Identified strategies to survive with these many stresses encompass responses by individual species, with the exception of biofilm formation which is often regarded as a community response. Commonly observed physiological characteristics of acidophiles are chemolithotrophy, iron respiration, obligate and facultative autotrophy, diazotrophy, osmotolerance, enhanced abilities to tolerate high concentrations of cationic transition metals and metalloids and a tendency to produce extracellular polymeric substance (EPS). Few extreme acidophiles, i.e. archaea, show thermotolerance, and some Gram-negative bacteria may exhibit psychrotolerance. Many of these physiological characteristics (e.g. metal

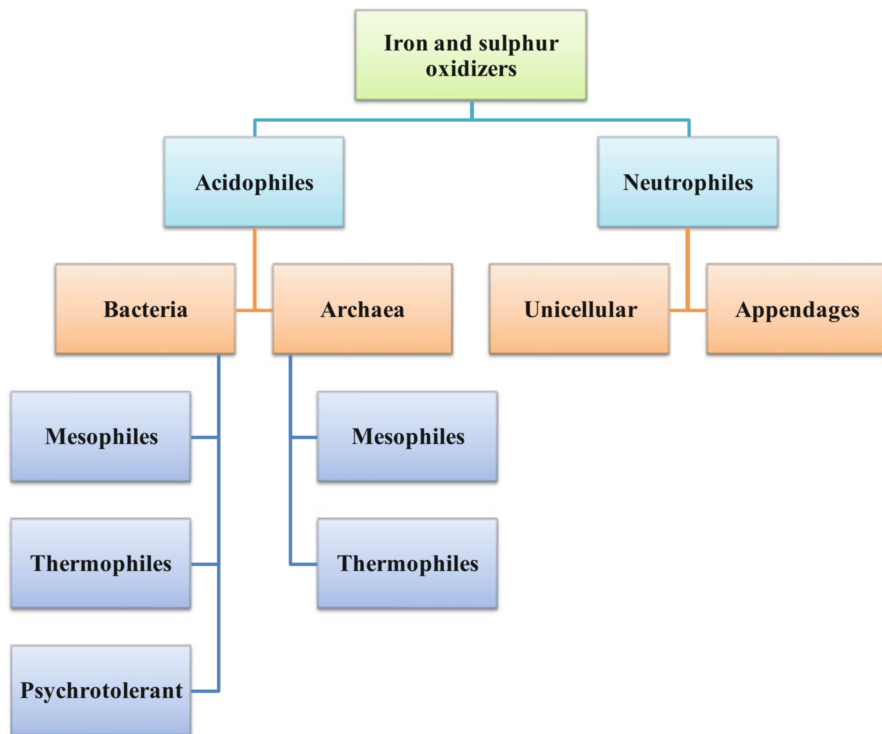


Fig. 10.1 Broad distribution of iron- and sulphur-oxidizing organisms based on growth temperature

tolerance) are globally conserved across acidophilic lineages, while others partition differentially between taxa like autotrophy and diazotrophy that indicate the unique feature of the microbial diversity of acidic environment (Denef et al. 2010).

Various metagenomic studies from natural and man-made acidophilic environment samples like acid mine drainage, mining tailings, commercial-scale heap leaching, mine adit, caves, acid salt lake, geothermal springs and concrete sewage system showed a wide diversity of acidophiles and their communities. These ecosystems showed the presence of taxa from the classes *Acidithiobacillia*; *α*-, *β*-, and *γ*-*Proteobacteria*; *Firmicutes*; *Actinobacteria*; *Nitrospirae*; *Aquificae*; *Verrucomicrobia*; Crenarchaeota; Thermoplasmata; unclassified Euryarchaeota; and candidates of Parvarchaeota. In the case of iron- and sulphur-oxidizing acidophiles, the reported genera of domain Bacteria are *Acidithiobacillus*, *Acidiferrobacter*, *Acidihalobacter*, *Thiomonas*, *Ferrovum*, *Acidiphilium*, *Acidocella*, *Acidomonas*, *Acidicaldus*, *Sulfobacillus*, *Alicyclobacillus*, *Acidibacillus*, *Desulfosporosinus*, *Acidithiomicrobium*, *Ferrimicrobium*, *Ferrithrix*, *Acidimicrobium*, *Acidithrix*, *Aciditerrimonas*, *Leptospirillum*, *Hydrogenobaculum* and *Methylacidiphilum*. Moreover, the noted genera of Archaea from the studied

natural and man-made ecosystems are *Sulfolobus*, *Stygiolobus*, *Metallosphaera*, *Acidianus*, *Acidilobus*, *Ferroplasma*, *Acidiplasma*, *Picrophilus*, *Cuniculiplasma*, *Thermoplasmatales*, *Thermogymnomonas*, *Aciduliprofundum*, *Parvarchaeum* and *Micrarchaeum*. The environmental temperature of the habitats in which these organisms are growing differentiates the prokaryotic community into bacteria and archaea (Quatrini and Johnson 2018; Dopson 2016, Golyshina et al. 2016, Sadeghi et al. 2016). Phylogeny of prokaryotic 16S rRNA genes and archaea from AMD samples is described in detail by Baker and Benfield (2003), where they have grouped prokaryotes in 11 putative divisions illustrating iron- and sulphur-oxidizing organisms along with other acidophilic bacteria of phyla *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Nitrospirae* and *Proteobacteria*. Archaeal lineages described by them are restricted to Thermoplasmatales and Sulfolobales. Several clones phylogenetically diverted from *Thermoplasma* are characterized, and they are grouped. As many as 14 clones are characterized, and these lineages are proposed as 'alphabet plasma'; even in *Ferroplasma*, only two species are cultured, and the rest of the 17 clones obtained may represent uncultured organisms.

More than 100 cultures from the BGR-strain collection centre was analysed by Breuker et al. (2009), and they found that most of the cultivable iron oxidizers are the members of genera *Acidithiobacillus*, *Acidimicrobium*, *Ferrimicrobium* and *Leptospirillum.* All the identified *Acidithiobacillus* strains are *A. ferrooxidans* with more than 98.5% similarity; in spite of that, they are from quite different mine waste dumps from various countries and from various geographic locations. Other Gram-negative iron oxidizers are identified as *L. ferriphilum* or *L. ferrooxidans.* These isolates are also less diverse phylogenetically as compared to the isolates of Gram-positive strains related to *Acidimicrobium* or *Ferrimicrobium* (Breuker et al. 2009).

Acidophilic members of AMD show distinct diversity in terms of temperature variation in the ecosystem. If the AMD temperature is below 30 °C, species of *Thiobacillus*, *Acidithiobacillus* and *Acidiphilium* are most active; when the temperature ranges between 30 and 50 °C, *Ferroplasma*, *Leptospirillum*, *Sulfobacillus* and *Eukaryotes* take the charge of the activities; and when the temperature rises above 50 °C, species of *Metallosphaera*, *Sulfobacillus* and *Thermoplasma* play the main role in the process. These organisms take part in iron oxidation, nitrogen fixation, sulphur oxidation and removal of organic matter in the sequence in each temperature section (Baker and Banfield 2003).

Metagenomic studies revealed the distribution of archaea and bacteria in acidic environments is uneven. It showed that due to anthropogenic activity (particularly biomining), > 75% dominance is of Bacteria, whereas, in the natural environments like hot springs, hydrothermal vents and other ecosystems, > 70% dominance is of Archaea. In both natural and anthropogenic extremely acidic environments, levels of species richness and evenness are considerably lower than those of non-extreme or non-acidic environments. Metagenomic studies have shown that irrespective of the specific habitat type, relatively few bacterial and archaeal species account for the vast bulk of acidic microbiomes (Cardenas et al. 2016; Ziegler et al. 2013; Jones et al. 2012).

10.3.1 Interactions Among Acidophiles

Acidophiles also show several kinds of interactions with each other like other microorganisms present in non-extreme ecosystems; some of these interactions are shown in Fig. 10.2. Positive interactions like synergism, mutualism and commensalism are seen, for example, between *Acidithiobacillus thiooxidans* and *Ferrimicrobium acidiphilum*, *Acidithiobacillus caldus* and *Sulfobacillus* sp. and *Acidithiobacillus ferrooxidans* and *Acidiphilum* sp., respectively. On the other hand, negative interactions, namely, competition, antagonism, predation and parasitism, are well documented among *Acidithiobacillus ferrooxidans* and *Leptospirillum* sp., *Ferroplasma acidiphilium* and *Leptospirillum ferriphilum*, Flagellates and *Acidiphilium* sp. and *Thermoplasma* sp. and Archaeal Richmond Mine acidophilic nanoorganisms (ARMAN) archaea, respectively. In order to enhance the efficiency of leaching of minerals and prevention and control of AMD generation and for biotechnological application of these organisms, it is very essential to know the relationship between the composition of microbial community, their function and interaction, but still few studies are available about it (Johnson 2016; Quatrini and Johnson 2018).

10.3.2 The Diversity of Major Iron- and Sulphur-Oxidizing Prokaryotes Among Acidophiles

A. ferrooxidans and *L. ferrooxidans* are the well-studied biomining bacteria reported to play a significant role in AMD generation and biomining. Currently, the genus *Acidithiobacillus* of the class *Acidithiobacillia* in phylum *Proteobacteria* is

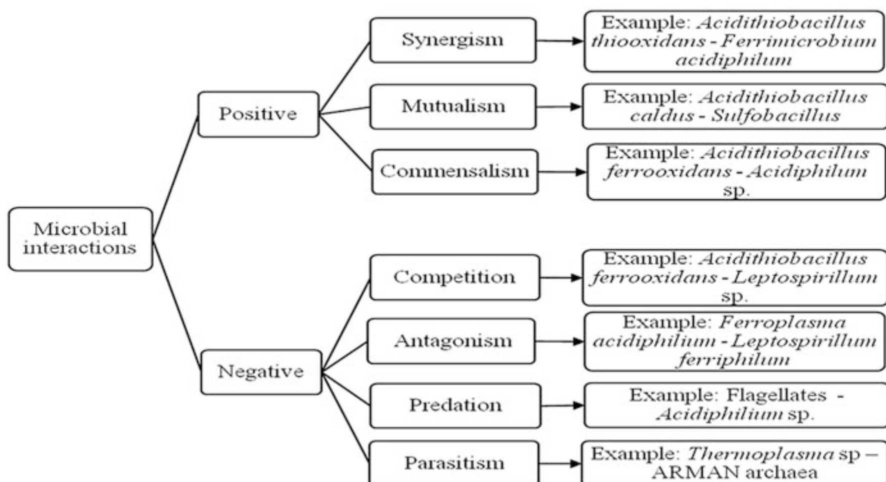


Fig. 10.2 Microbial interactions among acidophiles with examples

composed of seven valid species (described between the year 1922 and 2015), namely, *Acidithiobacillus thiooxidans*, *A. ferrooxidans*, *A. albertensis*, *A. caldus*, *A. ferrivorans*, *A. ferridurans* and *A. ferriphilus*. However, a large number of *Acidithiobacillus* strains and sequence clones have been obtained from a variety of ecological niches over the years, and many isolates are thought to vary in phenotypic properties and cognate genetic traits. However, after the introduction of molecular study, many more organisms are characterized directly from their ecosystem samples. Even if we consider *A. ferrooxidans*, to date >500 isolates are obtained, out of which only eight isolates' whole genome has been sequenced. Among these the complete genome sequence of strains ATCC 23270 and ATCC 53993 is published in 2006 and 2008, respectively. Six incomplete genome sequences are published till date; one of these, GGI-221, is isolated from an Indian mine; and an isolate from our laboratory is published in the year 2011 and is well referred by Zhang et al. (2018). Single circular genomes of these eight strains varied from 2.98 to 4.18 MB in size and G + C content is 57–59%, characterized minimum proteins are 2755 and the maximum is 4007. They also differ in terms of rRNA and tRNA reported. Based on the proteomic study, it is observed that when the ferrous iron is present as an energy substrate, the proteins are upregulated, but when sulphur is present as energy substrate, expression of these proteins is downregulated. With the randomly amplified polymorphic DNA (RAPD) study, 12 strains of *A. ferrooxidans* were grouped in 5 homogeneous groups, but similarities between these strains were as low as 5.49% and the maximum was 85.14%. The observed diversity could be explained as all these are isolated from geologically different locations. Much more diversity among *A. ferrooxidans* is well documented by Zhang et al. (2018). When metabolic activity is considered, these isolates showed a wide range of iron oxidation rate, metal leaching and tolerance to ferrous iron, ferric iron, metals, pH, temperature, salinity, organic substance and ionic strength. Bioleaching-process variability of *A. ferrooxidans* reported by 20 different groups in the last 20 years is also summarized by Zhang et al. (2018). Rajpardi lignite mine isolate *Acidithiobacillus ferrooxidans* strain SRDSM2 showed growth and iron oxidation in presence of 0.5 g/L peptone and 1.0 g/L tryptone soya broth and even tolerated temperature of 80 °C for 120 min (Patel et al. 2011).

The second most significant iron oxidizer after genus *Acidithiobacillus* is *Leptospirillum*; *Leptospirillum* means 'a thin spiral', a bacteria classified under phylum *Nitrospirae*, family *Nitospiraceae* and genus *Leptospirillum*. The members of genus *Leptospirillum* possess a Gram-negative cell wall, non-spore former vibrioid- or spiral-like shaped, and have an average of 0.9–2.0 × 0.2–0.5 μm cell size. The bacterium is commonly motile with a polar flagellum. The *Leptospirillum* are obligate chemolithotrophic as gain sole energy and reducing power through the oxidation of ferrous iron or ferrous iron-containing sulphidic minerals like pyrite. They are autotrophic as they utilize carbon dioxide as a carbon source. They fix carbon dioxide by the Benson-Calvin cycle. *Leptospirillum* is an obligate aerobe so the final electron acceptor is oxygen. They are acidophilic with optimum growth pH between 0.3 and 2.0. Majority of strains are mesophilic except for a single moderately thermophilic isolate which has been described. *Leptospirillum* forms a distinct lineage within the proposed *Nitrospirae* phylum (Hippe 2000). The mol% G + C of

the DNA is 51–56%. *Leptospirillum* are widely distributed in natural and industrial environments where the enhanced oxidation of sulphide ores creates mine water which is highly acidic and metal- and ferric-rich ecosystems. Commonly, *Leptospirillum* species have few distinct physiological characteristics which make them identifiable, but most researchers use molecular analyses to differentiate and identify them from other bacteria. The first documentation and type species *Leptospirillum ferrooxidans* was given by Markosyan in 1972 (Markosyan 1972; Sand et al. 1992). These extremely acidophilic leptospirilli display large physiological and genetic difference and according to 16S rRNA phylogeny have been classified into four species groups, namely, group I, *Leptospirillum ferrooxidans*; group II, *L. ferriphilum* and *Leptospirillum rubarum*; group III, *L. ferrodiazotrophum*; and group IV, UBA BS; recently, metagenomic evidence has supported the recognition of a new species ascribed in this group (Aliaga Goltsman et al. 2013; Zhang et al. 2018). Each member of this group is a strict (obligate) chemolithoautotrophic bacterium capable of assimilation of inorganic form of carbon, solely deriving energy from the aerobic oxidation of iron (Zhang et al. 2018). Due to this, they are some of the most metabolically restricted bacteria known. *Leptospirillum* sp. of iron-oxidizing bacteria plays an important role in industrial bioleaching and biooxidation processes, particularly reactor bioleaching processes. The reason for their domination in biooxidation tanks is that they are quite resistant to high ferric concentration, as prevailing high ferric-ferrous iron ratio inhibits all but species of *Leptospirillum*. They are widespread members of acidophilic microbial communities that catalyse ferrous iron oxidation, thereby aggravating sulphide mineral dissolution rates above 20 °C (as at lower temperature *Acidithiobacillus* spp. dominate). These organisms also play an important role in environmental acidification particularly AMD (Dave and Tipre 2012). Therefore, these reasons make leptospirilli more important microbes in the acid mine processes than previously thought. With reference to AMD, *Leptospirillum* is associated to contributing to pollution, and the noticeable example has been the issue of Iron Mountain in Northern California. It is the largest amount of toxic metal pollution in the United States and the rivers and streams that run off of the mountain contain very acidic waters (Druschel et al. 2004).

As can be seen from available literature, about 73 species of iron and sulphur oxidizers are described and grouped based on their characteristics in domains Bacteria and Archaea; it is very difficult to explain their diversity in one chapter. Thus, the diversity of various iron- and sulphur-oxidizing organisms is summarized briefly in the following section.

The major genera of iron- and sulphur-oxidizing Bacteria and Archaea that are reported in acidic environments and are of immense significance are shown in Table 10.1. Nearly 45 iron- and sulphur-oxidizing species are included in domain Bacteria, and out of these, 20 species oxidize both iron and sulphur/RISCs, whereas 13 are reported for iron oxidation and 12 for sulphur/RISCs only. These organisms are found in seven phyla, namely, *Actinobacteria*, *Aquificae*, *Firmicutes*, *Nitrospirae* and *α-β-γ-Proteobacteria*. Organisms oxidizing ferrous iron but not reducing sulphur/RISCs are distributed in all these phyla except *Aquificae*. These bacteria are diverse in terms of pH, temperature, nutrition requirements and tolerance to various

Table 10.1 The diversity of iron- and sulphur-compound-oxidizing bacteria found in natural and man-made ecosystems (Schippers 2007; Dopson 2016; Golyshina et al. 2016)

Phylum	Species	Phylum	Species
Actinobacteria	*Acidimicrobium ferrooxidans*	Firmicutes	*Alicyclobacillus disulfidooxidans*
	Acidithrix ferrooxidans		*Alicyclobacillus tolerans*
	Ferrimicrobium acidiphilum		*Alicyclobacillus ferrooxydans*
	Acidithiomicrobium sp.		*Alicyclobacillus aeris*
	Ferrithrix thermotolerans		*Caldibacillus ferrivorus*
α-Proteobacteria	*Acidithiobacillus albertensis*		*Sulfobacillus acidophilus*
	Acidithiobacillus caldus		*Sulfobacillus montserratensis*
	Acidithiobacillus ferrooxidans		*Sulfobacillus sibiricus*
	Acidithiobacillus thiooxidans		*Sulfobacillus thermosulfidooxidans*
	Acidithiobacillus ferrivorans		*Sulfobacillus thermotolerans*
	Acidithiobacillus ferriphilus		*Sulfobacillus benefaciens*
	Acidithiobacillus ferridurans		*Acidibacillus ferrooxidans*
	Acidiphilium acidophilum		*Acidibacillus sulfuroxidans*
	Acidiphilium sp.	Nitrospira	*Leptospirillum ferriphilum*
	Acidiphilium rubrum		*Leptospirillum ferrodiazotrophum*
	Acidicaldus organivorans		*Leptospirillum ferrooxidans*
β-Proteobacteria	*Thiobacillus plumbophilus*		*Leptospirillum rubarum*
	Thiomonascuprina		*Leptospirillum* sp. group IV
	Thiomonas arsenitoxydans	Aquificae	*Hydrogenobaculum acidophilum*
	Thiomonas intermedia		*Hydrogenobaculum* sp.
	Ferrovum myxofaciens		
ϒ-Proteobacteria	*Acidiferrobacter* sp.		
	Acidiferrobacter thiooxydans		
	Acidihalobacter ferrooxidans		
	Acidihalobacter prosperus		

Iron oxidizers (Fe^{2+}); sulphur oxidizers (S^0/RISCs); iron and sulphur oxidation (both); RISCs: reduced inorganic sulphur compounds

extreme environments. *Acidiphilium* spp. are obligate, aerobic, chemoorganotrophic (an exception is *A. acidophilum* that shows autotrophy at oxidizing elemental sulphur and RISCs), and major species can withstand pH 2.0–5.9 and are more tolerant to chloride than other acidophilic bacteria. *Thiobacillus prosperus* and related bacteria are Gram-negative, mesophilic, halophilic acidophiles that oxidize sulphur and iron. Many species of genus *Acidithiobacillus* grow below pH 4.0; they are mesophilic chemolithotrophic Gram-negative rod-shaped bacteria comparatively more sensitive to NaCl and can oxidize sulphur and/or iron. In contrast to *Acidithiobacillia*, *Leptospirillum* spp. are extremely acidophilic vibrioid/spirillum-

Table 10.2 The diversity of iron- and sulphur-compound-oxidizing archaea found in natural and man-made ecosystems (Schippers 2007; Dopson 2016; Golyshina et al. 2016)

Phylum	Species	Phylum	Species	Phylum	Species
Euryarchaeota	*Ferroplasma acidarmanus*	Crenarchaeota	*Sulfolobus shibatae*	Crenarchaeota	*Metallosphaera hakonensis*
	Ferroplasma acidiphilum		*Sulfolobus tokodaii*		*Metallosphaera prunae*
	Ferroplasma sp. type II		*Sulfolobus yangmingensis*		*Metallosphaera sedula*
	Ferroplasma thermophilum		*Sulfolobus tengchongensis*		*Metallospaera cuprina*
	Ferroplaera cupricumulans		*Sulfurisphaera ohwakuensis*		*Metallospaera yellowstonensis*
	Acidiplasma cupricumulans		*Acidianus brierleyi*		*Sulfolobus metallicus*
	Acidiplasma sp.		*Acidianus infernus*		*Sulfolobus yangmingensis*
			Acidianus hospitalis		*Sulfurococcus mirabilis*
			Acidianus manzaensis		*Sulfurococcus yellowstonensis*
			Acidianus sulfidivorans		*Acidianus ambivalens*
			Acidianus copahuensis		

Iron oxidizers (Fe^{2+}); sulphur oxidizers (S^0/RISCs); iron and sulphur oxidation (both); RISCs: reduced inorganic sulphur compounds

like-shaped chemolithotrophic iron-oxidizing bacteria. *Sulfobacillus* spp., the iron- and sulphur-oxidizing moderately thermophilic acidophiles, can grow auto- or heterotrophically. Many species show significant tolerance towards NaCl. The acidophilic, thermotolerant, iron- and sulphur-oxidizing bacteria *Acidiferrobacter thiooxidans* are reported to be a moderate osmophile.

Acidophilic archaea represent 28 species; they are found in phyla Euryarchaeota and Crenarchaeota. Among the 28 organisms, 11 have only sulphur/RISCs-oxidizing ability, and 7 species are found to utilize only iron, whereas 10 are able to grow using iron, sulphur or reduced sulphur compounds (Table 10.2). Most of these are more tolerant to salts than their counterpart bacteria, but large variations in their tolerance to salts are noted. *Acidianus* spp. are autotrophic, facultative aerobes, oxidize reduced sulphur and iron depending on oxygen availability and show a very wide range in growth, pH and temperature. On the other hand, species of *Sulfolobus* and *Thermoplasma* are thermophilic sulphur oxidizers. *Ferroplasma* spp. playing a significant role in AMD and bioleaching are extremely acidophilic but moderately thermophilic to mesophilic iron oxidizers (Schippers 2007; Dopson 2016; Golyshina

et al. 2016). A novel thermoacidophilic iron- and sulphur-oxidizing archaeon strain YN25 is isolated by Ding et al. (2011) having an optimum growth temperature of 65 °C and pH 1.5–2.5; it grows heterotrophically in the presence of several organic substances. The isolate is named as *Acidianus manzaensis* YN25.

10.3.3 Significance of Acidophiles with Special Reference to Biomining

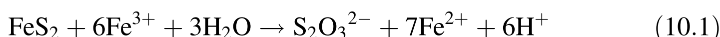
The biotechnological potential of chemolithoautotrophic iron- and sulphur-oxidizing acidophiles has been exploited in commercial-scale biomining operations for the last 50 years, and still, new openings for developing 'biomining' technologies are emerging (Johnson 2014). Today, biohydrometallurgy is a gradually evolving technology because it efficiently extracts metals from low-grade ores and tailings, minerals, concentrates and even e-waste in the era of depleting natural resources. The microbial communities are one of the crucial factors influencing the leaching productivity; the selection of highly efficient microorganisms or microbial communities is the vital criteria to increase the bioleaching rates or efficiencies of the process (Johnson 2008). Various studies are reported on the isolation of most efficient strains and developing consortia or mixed cultures (Liu et al. 2011; Zhang et al. 2015; Xiao et al. 2016). However, these measures appeared to be a little blind and unpractical in bioleaching industry, because it seems impossible to build appropriate microbial community before understanding the composition and function of in situ microbial communities in bioleaching systems. Therefore, it is important to put insight into the composition, structure and function of microbial communities in biological heap leaching system and into the relationships between composition and function of microbial communities in the bioleaching system.

10.3.4 Bioleaching of Metals from Minerals

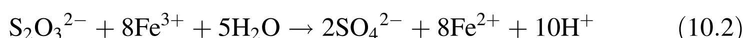
Selected iron- and sulphur-oxidizing microorganisms are used at industrial scale in the form of dump, heap and reactor processes for commercial-scale leaching of copper, cobalt, gold and uranium from their ore or concentrates (Rawlings 2005). In metal bioleaching process, iron- and sulphur-oxidizing organisms provide the required oxidizing agent, protons and space of reactions by exopolysaccharide (EPS) layer formation (Gehrke et al. 1998; Rohwerder et al. 2003; Sand et al. 1995); thus the chemical reaction of metal mobilization takes place. Minerals to be amenable to bioleaching process should have the presence of iron and reduced sulphur compounds; otherwise these compounds need to be added from outside.

Metal solubilization from the minerals is mainly divided into two mechanisms. In case of pyrite and molybdenite, thiosulphate - the mechanism is functioning where acid-insoluble metal sulphides are leached out as depicted in Eq. 10.1 and 10.2. *A. ferrooxidans*, *L. ferrooxidans*, *L. feriphillum* and species of *Sulfobacillus* play a significant role by oxidizing the ferrous iron produced (Eq. 10.1 and 10.2) to ferric

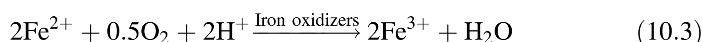
iron (Eq. 10.3). In this process, thiosulphate is the main intermediate produced, which is further oxidized by ferric iron into sulphate as an end product (Rohwerder et al. 2003). The generated ferrous iron due to solubilization of metals needs to be regenerated to ferric iron for the cyclic process. The iron-oxidizing microorganisms attack the produced ferrous iron and generate the required ferric iron for the next cycle of metal solubilization.



$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow S_2O_3{}^{2-} + 7Fe^{2+} + 6H^+ \quad (10.1)$$



$$S_2O_3{}^{2-} + 8Fe^{3+} + 5H_2O \rightarrow 2SO_4{}^{2-} + 8Fe^{2+} + 10H^+ \quad (10.2)$$

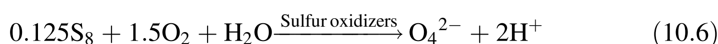


$$2Fe^{2+} + 0.5O_2 + 2H^+ \xrightarrow{\text{Iron oxidizers}} 2Fe^{3+} + H_2O \quad (10.3)$$

The second is polysulphidic mechanism responsible for metal extraction from acid-soluble minerals like chalcopyrite, galena and sphalerite by the synergistic action of ferric iron and protons (Eqs. 10.4, 10.5 and 10.6). In this process, sulphur-oxidizing organisms such as *A. thiooxidans* and *Acidithiobacillus caldus* oxidize elemental sulphur to sulphate and generate protons (Rawlings 2005). The required ferric ions are generated by the iron-oxidizing organisms. The ferrous iron generated during the process of any of the two mechanisms is biooxidized by iron oxidizers as shown in Eq. 10.3. If the mechanisms are summarized in simple words for biological action than it can be said that function of iron- and sulphur-oxidizing organisms is generation of sulphuric acid as require protons for the metal solubilization from acid-soluble minerals and to keep produce ferric iron in solution in soluble form and ferric iron as lixiviants for metal extraction from both the types of minerals.

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5H_2S_n + Fe^{2+} \ (n \geq 2) \quad (10.4)$$

$$0.5H_2S_n + Fe^{3+} \rightarrow 0.125S_8 + Fe^{2+} + H^+ \quad (10.5)$$



$$0.125S_8 + 1.5O_2 + H_2O \xrightarrow{\text{Sulfur oxidizers}} O_4{}^{2-} + 2H^+ \quad (10.6)$$

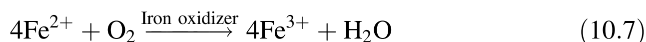
The heap-, dump- or reactor-based bioleaching processes operate broadly at two temperatures. Irrespective of the types of minerals used, if the process is operated from ambient to 40 °C temperature, mesophilic organisms are the key biocatalyst for the process. If the process is operated between 45 and 55 °C or 75 and 80 °C, thermophilic iron and sulphur oxidizers will take the charge of the process (Rawlings et al. 2003). When the process is operated up to 40 °C temperature, a consortium consisting of Gram-negative bacteria mainly consisting of *A. ferrooxidans*, *A. thiooxidans*, *A. caldus*, *L. ferrooxidans* and *L. ferriphilum* play a pivotal role (Coram and Rawlings 2002; Foucher et al. 2003; Goebel and Stackebrandt 1994; Hallberg and Lindström 1994; Vásquez and Espejo 1997). Apart from these five organisms, Gram-positive *Sulfobacillus thermosulfidooxidans* and heterotrophic acidophilic species of genera *Acidiphilium* and *Ferroplasma* are also detected from the process samples (Foucher et al. 2003; Golyshina et al. 2000; Harrison 1981). When the leaching process temperature is >70 °C, instead of iron- and

sulphur-oxidizing bacteria, members of Archaea dominate the process, and species of *Sulfolobus* and *Metalloshaera* play a lion share of activity in metal solubilization (Norris 1997; Norris et al. 2000).

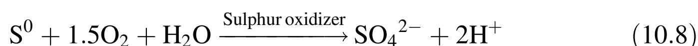
Solubilization of more than 80% of copper, zinc and lead from polymetallic ore and concentrates is reported by application of *A. ferrooxidans*, *L. ferrooxidans*, *L. ferriphilum* or a consortium of these iron- and sulphur-oxidizing organisms in leaching processes performed in a shake flask as well as stirred tank reactor (Tipre and Dave 2004; Patel et al. 2012a,b). Dong et al. (2013) have shown the utility of *A. ferrooxidans* for the extraction of copper from djurleite, bornite, covellite, chalcopyrite, etc.

10.3.5 Bioleaching of Metals from E-Waste

A huge amount of e-waste is generated throughout the world. It is increasing fast and at an uncontrolled rate, it is the fastest growing pollutant as a fraction of solid waste. All the e-waste contains one or the other metal in their printed circuit boards (PCBs). Copper is the major metal in almost all PCBs. Metals present in e-waste are in the form of their zero valences. To solubilize them from e-waste using microbes, it is necessary to add ferrous iron, which provides an energy source for iron oxidizers, and bio-generated ferric iron by the iron oxidizers is used for leaching of copper, zinc, nickel, etc. from the waste. In e-waste, the main lixiviant required for metal solubilization is ferric iron. *A. ferrooxidans*, *A. thiooxidans*, *L. ferrooxidans* and *L. ferriphilum* are found to play a significant role in the process (Saidan et al. 2012; Dave et al. 2018; Sodha et al. 2017). The two-phase process developed for e-waste is the method of choice due to more and fast solubilization of metals from e-waste using higher pulp density of e-waste in the process (Shah et al. 2015; Sodha et al. 2018). In the case of several e-wastes, it is not necessary to pulverize the waste, and the PCBs can be treated without grinding. Both in the case of pulverized and non-pulverized e-waste in general and PCBs in particular, it is necessary to give pretreatment to remove epoxy layer for the improved activity of bio-generated ferric iron and protons (Dave et al. 2018, Chauhan et al. 2018; Sodha et al. 2018). The metal solubilization can be represented by Eqs. 10.7, 10.8, 10.9, 10.10, 10.11 and 10.12, where iron- and sulphur-oxidizing organisms produce ferric iron and protons as shown in Eqs. 10.7 and 10.8, respectively. These produce lixiviants that act on metals present on PCBs and oxidize them and give their respective sulphate salts (Dave et al. 2018, Chauhan et al. 2018).

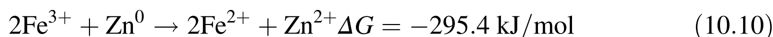


$$4Fe^{2+} + O_2 \xrightarrow{\text{Iron oxidizer}} 4Fe^{3+} + H_2O \tag{10.7}$$

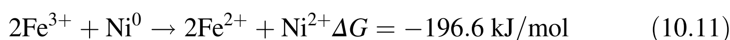


$$S^0 + 1.5O_2 + H_2O \xrightarrow{\text{Sulphur oxidizer}} SO_4{}^{2-} + 2H^+ \tag{10.8}$$

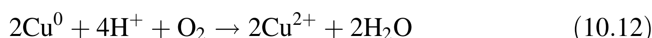
$$2Fe^{3+} + Cu^0 \rightarrow 2Fe^{2+} + Cu^{2+} \Delta G = -82.9\ \text{kJ/mol} \tag{10.9}$$



$$2Fe^{3+} + Zn^0 \rightarrow 2Fe^{2+} + Zn^{2+} \Delta G = -295.4\ \text{kJ/mol} \quad (10.10)$$



$$2Fe^{3+} + Ni^0 \rightarrow 2Fe^{2+} + Ni^{2+} \Delta G = -196.6\ \text{kJ/mol} \quad (10.11)$$



$$2Cu^0 + 4H^+ + O_2 \rightarrow 2Cu^{2+} + 2H_2O \quad (10.12)$$

10.3.6 Production of Pigments from Acid Mine Drainage

Iron-oxidizing acidophilic microorganisms have been used to produce iron oxide pigments from AMD. Irrespective of the reaction pH, the presence of acidophilic iron oxidizers enhances the pigment production by 30-fold as compared to their absence (Murphy 2017). The iron oxide pigments generated from AMD can be used as paint and colouring brick, block and cement used in construction due to their optical properties, ready availability and cost-effectiveness. However, the formation of jarosite may be responsible for the inferior quality of the pigment; thus future optimization to get desired pigments for better quality is needed.

10.3.7 Desulphurization

Apart from metal extraction from tailing, ore, concentrate and e-waste, *A. ferrooxidans*, *A. thiooxidans* and other iron, as well as sulphur-oxidizing organisms, are effectively used to remove sulphur from coal, waste rubber and gas based on its oxidizing capacity of ferrous iron or reduced sulphur compounds (Dong et al. 2013). Desulphurization improves the quality of the minerals and reduces environmental pollution when they are burnt. This technology is extensively used for desulphurization of coal.

10.3.8 Removal of Iron from Acidic Mine Water

We have seen the role of iron- and sulphur-oxidizing organisms in AMD formation. The same activity of iron-oxidizing organisms is beneficially used for the precipitation of iron from the acidic water. Ferrous iron is highly soluble in acidic water, and if it is to be removed by precipitation, the pH of the system needs to be raised to 8–9, which requires a large amount of alkali. Instead of this, if ferrous iron present in the acidic water is oxidized by the use of iron-oxidizing organism, the generated ferric iron can be easily precipitated out by rising pH to about 5. This saves the amount of alkali and also gives better remediation. Moreover, the precipitated ferric iron may be used as a pigment or a colouring agent.

10.3.9 Acid Mine/Rock Drainage Generation

Iron- and sulphur-oxidizing organisms have both beneficial and adverse effects at mining sites. The adverse effect is the formation of acidic drainage. Due to the combination of chemical and biological processes, mine water gets polluted in terms of acidity, a high concentration of metal ions, turbidity as well as precipitates of ferric iron. Biological acid mining activity will start with the involvement of neutrophilic sulphur-oxidizing microorganisms such as *Thiobacillus thioparus*, *Thiobacillus neopolitanus*, *Thiobacillus denitrificans*, etc. These organisms initiate oxidation of sulphur or RISCs and bring down the pH to ~5.0. Once the pH of the AMD reaches 5 or below 5.0, acidophilic iron and sulphur oxidizers will get activated, and more amounts of H^+ are produced which results in further reduction in pH that may be as low as below 1.0. Lignite mines drainage of Rajpardi, Tadkeshwar and Panandhro, Gujarat, India, showed pH below 3.0 (Patel et al. 2009). For example, the picture of Tadkeshwar lignite mine situated in Gujarat, India (Fig. 10.3a), clearly indicates the accumulation of acidic water of pH below 3.0. It also shows the precipitation of ferric iron. The amount of acid or ferric iron precipitates generated at mine sites depends on physical, chemical and biological factors at those sites. The extensively studied mechanism of AMD formation is oxidation of pyrite in the presence of ferric iron, where the oxidant ferric iron is generated by the activity of species of *Acidithiobacillus* and *Leptospirillum*. Initially, pyrite is chemically oxidized and creates favourable condition for the activity of *Acidithiobacillus ferrooxidans*, which is further dominated by *Leptospirillum ferrooxidans* and *L. ferriphilium*. Owing to the presence of these organisms, the oxidation rate of pyrite increases to 5×10^5 to 1×10^6 folds (Kelly and Tuovinen 1988; Brierley and Brierley 2002). As studied by Patel et al. (2011), the volume of acidic water generated at Rajpardi lignite mine (situated in Bharuch district, Gujarat, India) was $>10 \times 10^6$ m^3 which was as acidic as having pH ~2.0 (Fig. 10.3b). Due to the occurrence of gangue material at mining sites and in minerals, other water-soluble pollutants are also mixed in AMD. In several

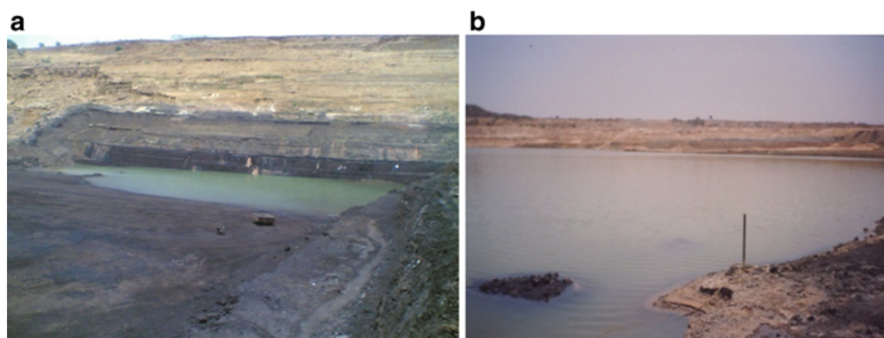
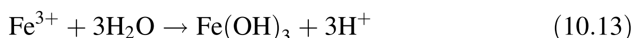


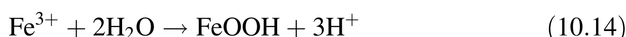
Fig. 10.3 Acidic mine drainage sump of lignite mine, Gujarat, India. (**a**) Tadkeshwar (**b**) Rajpardi lignite

Fig. 10.4 Acid mine drainage of sulphide mine, at Malanjkhand, Madhya Pradesh, India, showing ferric iron and copper sulphate precipitation

instances, due to the low solubility, ferric iron gets precipitated. These precipitates are known as 'yellow boy', 'boulder coats' or 'ochres' as shown in Eqs. 10.13 and 10.14.



$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+ \quad (10.13)$$



$$Fe^{3+} + 2H_2O \rightarrow FeOOH + 3H^+ \quad (10.14)$$

Due to the acidity of the AMD in sulphide metal mines, various metal ions get solubilized. The generation and solubilization of copper are clearly seen at metal sulphide mine in Fig. 10.4. Because of this, the environment becomes more extreme and toxic for the survival of another group of organisms and other fauna and flora due to this large portion of the nearby creeks and surrounding environments get sterilized in terms of living creature other than acidophilic metal tolerant organisms. Even abandoned mines are generating acidic metal-containing drainage, for example, some Roman mining sites in Great Britain closed 2000 years ago still generate contaminated acidic water (Coil et al. 2014). An extremely low pH of 3.6 has been reported at the Iron Mountain Mine in California, which is approximately 1000 times more acidic than the battery acid (Coil et al. 2010).

10.3.10 Future Perspectives

Microbial cultivation techniques and media need to be developed for the cultivation of these organisms. Ecological functions and community structure in diverse AMD gradients can be investigated in further detail. The influence of various depths of mine and ecological gradients on microbial community requires to be studied to understand the importance of functions occurring at various depths. The role of many iron- and sulphur-oxidizing members is still not properly known, which is required for an accurate understanding of the microbial ecology so it can be exploited for other biotechnological applications.

10.4 Conclusions

Microorganisms play a significant role in the generation of AMD. Acidophilic iron- and sulphur-oxidizing organisms are highly diverse in terms of their habitats, requirement of energy and carbon source, optimum pH and temperature for growth and metabolic activities. They show both synergistic and antagonistic relations among their groups. They are responsible for the global environmental problem of acid mine/rock drainage at active as well as abandoned mines, many hundreds of years after mining activity has stopped. If these organisms are properly harnessed and exploited, they turn out to be efficient biotechnological tools for metal extractions, desulphurization, pigment production and bioremediation of iron from acidic waste. The use of molecular techniques and community study carried out in the last two decades significantly improved our knowledge on the diversity and community function of acidophilic iron and sulphur oxidizers in AMD ecosystems.

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11 Distribution and Bioprospecting Potential of Actinobacteria from Indian Mangrove Ecosystems

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Abstract

Actinobacteria are the ecologically and economically important prokaryotes with the unprecedented ability to produce novel metabolites. They are widely distributed in nature due to their physiological and metabolic versatility. Actinobacteria from marine ecosystem are recognized as a promising source for novel bioactive metabolites. Notably, mangroves are the most fertile and productive marine ecosystem due to their ecophysiological conditions and vast microbial diversity. At the global level, there are many novel actinobacteria which have been reported from mangrove ecosystems and also explored for their bioprospecting potential. However, in India, microbiologists have explored actinobacteria from selected mangrove ecosystems for the past five decades with special reference to culturable diversity and antimicrobial, antimycobacterial, anticancer, and enzymatic activities. So far, around 22 rare actinobacterial genera have been reported from the Indian mangrove ecosystems. However, several other novel actinobacteria with promising bioprospecting potential are yet to be explored. Hence, research on actinobacteria in the Indian mangrove ecosystem is still in its infancy. This chapter will describe the exploratory research work carried out on the importance, diversity, antimicrobial and

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enzymatic activity, biodegradation, and nanotechnological potency of actinobacteria from the Indian mangrove ecosystems. This chapter will enable researchers to identify the gaps to be bridged for further research on Indian mangrove actinobacteria and exploit their potential to the maximum benefit to mankind.

Keywords
Actinobacteria · Distribution · Bioprospecting · Mangrove ecosystem · India

11.1 Introduction

Microorganisms are the pioneer colonizers of our planet which constitute the prime pool of biochemical pathways with prospective biotechnological and environmental implications. Microbes are incalculably important to the environment as well as to all life forms (Bisen et al. 2012). Over 3.8 billion-year-old microorganisms play key roles in the global cycling of nutrients, matter, and energy in our planet Earth, and they are proper master chemists with the unprecedented ability to perform various and complex chemical reactions (Salazar and Sunagawa 2017). Hence, they live in every bend of the atmosphere including the ocean where they are distributed in open waters, sediments, estuaries, hydrothermal vents, and bodies of marine animals and plants (Cevera et al. 2005). They are always involved in the important processes of the ocean in promoting organic material transformation and mineralization in the sediments and overlying waters (Das et al. 2007). Microbial communities are controlled by the spatiotemporal variability and physicochemical and biotic parameters (Hewson et al. 2007).

Microbes of both terrestrial and aquatic counterpart have proven to be a treasure house for new chemical entities (NCEs). Microbial bioprospecting programs have yielded several secondary metabolites notably antibiotics over the past 60 years (Busti et al. 2006). In agreement with the survey of a Business Communication Company (BCC), the global market for microbes and microbial products was expected to reach nearly $306 billion for compound annual growth rate (CAGR) of approximately 14.6% over the periods from 2015 to 2020 (Microbial Products 2015; Singh et al. 2017).

11.1.1 Actinobacteria

Actinobacteria are one of the major bacterial phyla comprised of Gram-positive bacteria with diverse morphological, physiological, and metabolic characteristic forms and DNA with high guanine + cytosine (G+C) composition. Most genera belonging to this phylum are free-living saprophytes that are universally found in both aquatic and terrestrial ecosystems. This phylum also includes several pathogens, such as species of *Mycobacterium*, *Corynebacterium*, *Nocardia*,

Fig. 11.1 Isolation of actinobacteria from mangrove sediments

Propionibacterium, and *Tropheryma*; soil inhabitants, such as *Streptomyces* spp. and *Micromonospora* spp.; plant commensals, such as *Frankia* spp.; and gastrointestinal commensals, such as *Bifidobacterium* spp. The existence of actinobacterial genera in space stations is also documented through culture-based and metagenomic approaches (Vaishampayan et al. 2013; Ichijo et al. 2016). Actinobacteria have a variety of morphologies (Fig. 11.1) ranging from rod-coccoid (e.g., *Arthrobacter* spp.) or coccoid (e.g., *Micrococcus* spp.) to highly differentiated mycelium (e.g., *Streptomyces* spp.) or fragmented hyphal forms (e.g., *Nocardia* spp.) (Barka et al. 2016). In general, soil microbial populations are dominated by the actinobacterial genus *Streptomyces*. Most of the actinobacterial genera are mesophilic in nature which means they grow optimally at temperatures between 25 and 30 °C at a neutral pH with the best growth pH between 6 and 9.

Actinobacteria are naturally present at densities in an order of 10^6–10^9 cells per gram of soil (Goodfellow and Williams 1983). However, their habitat and the prevailing climatic conditions influence their population density. Factors such as pH, temperature, and moisture content also impact the growth of actinobacteria. The first study on the effect of climate on the distribution of actinobacteria was done in 1903; Hiltner and Stromer, for the first time, observed that actinobacteria account for 20% of the microbial flora of the soil in spring, whereas in autumn, they account for more than 30% because of the huge amounts of crop residues available at this time of the year. However, frost reduces the relative abundance of actinobacteria to 13% during the winter.

The taxonomic position of the phylum actinobacteria is well proposed through the 16S rRNA sequencing. There are 6 classes in the phylum *Actinobacteria*, viz. *Rubrobacteria*, *Thermoleophilia*, *Coriobacteria*, *Acidimicrobiia*, *Nitriliruptoria*, and *Actinobacteria*, 5 subclasses, 6 orders, and 14 suborders (Ludwig et al. 2012). Till 2016, approximately 340 rare genera of actinobacteria (www.bacterio.net) have been discovered, but new taxa continue to be discovered as an ongoing process (Azman et al. 2017).

Actinobacteria, notably *Streptomyces*, are notorious for their ability to produce chemically unique secondary metabolites. Several actinobacterial secondary metabolites have been explored as a source of antibacterial, anticancer, antifungal, antiparasitic, and immunosuppressive agents (Baltz 2007; Berdy 2012). Notably, the diversity of chemicals in synthetic library has been exceeded by the chemically diverse secondary metabolites produced by this taxon. Actinobacterial metabolites have been preselected through millions of years of evolution to interact effectively with biological targets (Heul et al. 2018). The discovery of new active metabolites from this phylum has marked an era in antibiotic research as most of its antibiotics are too complex to be synthesized by combinatorial chemistry (Baltz 2007; Barka et al. 2016). Over the past 50 years, actinobacterial research has led to the finding of 50% of total antibiotics reported. However, only a few soil-derived genera such as *Streptomyces* and *Micromonospora* account for most of these compounds. Members of the genus *Streptomyces* are the most abundant producers of bioactive secondary metabolites accounting up to 80% of the bioactive small molecules discovered from actinobacteria. According to some estimates, the top 10 cm of global soil contains 10^{25}–10^{26} of actinobacteria, but only about 10^7 have been screened for antibiotic production in the last half-century, leaving plenty of scope for further screening (Baltz 2007).

Till the year 2000, only 20 new antibiotics have been launched worldwide, of which 11 were natural product-derived and 9 were synthetically derived (Butler and Cooper 2011). Among the 11 natural product-derived antibiotics, majorities were belonging to the beta-lactam class, and in particular, 8 were synthesized based on the antibiotics isolated from actinobacteria as a lead source. Meanwhile, further discovery of unknown metabolites from *Streptomyces* was predicted by the genome analysis. The numbers of metabolites actually isolated so far are less than the number of secondary metabolites isolated by biosynthetic gene clusters and identified in the whole genome of *Streptomyces avermetilis* and *Streptomyces coelicolor* (Igarashi et al. 2012). Out of the 33,500 microbial bioactive metabolites reported during the period of 1940–2010, about 13,700 were synthesized by actinobacteria mostly of terrestrial origin (Berdy 2012).

11.1.2 Marine Actinobacteria

Microbes play a critical role in structuring and functioning of the marine ecosystem. Giving remarkable diversity to the ocean microbiome, uncovering marine microbial taxa remains a fundamental challenge in microbial ecology. The heterogeneity of the ocean due to the presence of nutrient patches and microscale gradients results in different niches that can support diverse types of microbes. In addition, large-scale spatial and temporal variations in the ocean also contribute to environmental heterogeneity and can sustain microbial diversity (Gajigan et al. 2018).

It has long been recognized that actinomycetes can be cultured from marine samples (Grein and Meyers 1985; Goodfellow and Haynes 1984). The recovery of actinobacterial strains from deep-sea sediments (Weyland 1969), the report of the

Table 11.1 List of representative new rare actinobacterial species isolated from the marine ecosystem

Actinobacteria	Marine ecosystem
Amycolatopsis marina sp. nov.	South China Sea
Brevibacterium oceani sp. nov.	Mariana Trench (Challenger Deep)
Dermococcus bharathri sp. nov.	Mariana Trench (Challenger Deep)
Microbacterium indicum sp. nov.	Chagos Trench, Indian Ocean
Modestobacter marinus sp. nov.	Atlantic Ocean
Nesterenkonia alkaliphila sp. nov.	Western Pacific ocean
Pseudonocardia antitumoralis sp. nov.	South China Sea
Sciscionella marine gen. nov. sp. nov.	North-South China Sea
Serinicoccus profoundi sp. nov.	Indian ocean
Verrucosispora sediminis sp. nov.	South China Sea
Williamsia marianensis sp. nov.	Mariana Trench (Challenger Deep)

first marine species (Helmke and Weyland 1984), and the isolation of seawater-dependent strains (Jensen et al. 1991) further supported the existence of indigenous marine actinobacterial population. More recently, considerable actinobacterial diversity existing within marine samples has been explored by the cultivation efforts taken by several researchers (Magarvey et al. 2004; Jensen et al. 2005; Maldonado et al. 2005b; Gontang et al. 2007). It is now evidenced that actinobacteria are capable of growth in the marine environment (Moran et al. 1995; Jensen et al. 2005). The recent description of three marine genera provides strong support for the concept of marine-specific actinobacteria (Han et al. 2003; Yi et al. 2004; Maldonado et al. 2005a). Today, applying the culture-independent techniques to marine samples consistently revealed the existence of actinobacteria. Globally, about 21 new species of actinobacteria were isolated from the deep sea between the years 2005 and 2015 (Kamjam et al. 2017). In the last decade, there were several novel rare actinobacterial genera isolated from marine sediments and marine organisms (Table 11.1).

Among the different marine microbial phyla, actinobacteria produce the major fraction of metabolites with diverse biological activities such as antimicrobial, antiparasitic, anti-inflammatory, and anticancer activities (Abdelmohsen et al. 2017). In recent years there were several new metabolites discovered from marine actinobacteria as compared to actinobacteria from terrestrial ecosystems, even with the limited screening efforts. During the period of 2003–2005, about 23 novel metabolites were reported from marine actinobacteria (Lam 2006). During the last decade, around 659 marine bacterial compounds have been described, out of which 256 compounds have originated from actinomycetes (Williams 2009). Studies on compounds such as salinosporamide and abyssomicin isolated from the unique marine actinobacterial genera *Salinispora* and *Verrucosispora* suggest that these bacteria add an important dimension to marine natural product research (Sarkar et al. 2008). A recent review by Blunt et al. (2018) revealed that there are 179 new natural products isolated in the year 2016, out of which the actinobacterial genus *Strepto-myces* continues to be the dominant source. Some novel metabolites reported from

Table 11.2 List of novel metabolites reported from marine actinobacteria worldwide

Metabolites	Activity	Source	Ecosystem	References
Grisemycin	–	*Streptomyces griseus* M268	Kiaochow Bay, China	Xie et al. (2016)
Mohangic acid E	Anticancer	*Streptomyces* sp. SNM 31	Intertidal mudflats, Buan, Republic of Korea	Bae et al. (2016)
Ansalactams B-D	Anti-MRSA	*Streptomyces* sp. CNH189	–	Wilson (2011)
Bohemamines D-I	–	*Streptomyces* sp. SNB048	Sand sample, Bohmanian tidal flat	Fu et al. (2016)
Dibohemamines A-C	Anticancer	*S. spinoverucosus* SNB032	Sand sample, Bohmanian tidal flat	Fu et al. (2015)
Tetrocarcin N & O	Antibacterial	*Micromonospora* sp.	Sediment, Bohai Bay, China	Tan et al. (2016)
Micromonohalimane A & B	Anti-MRSA	*Micromonospora* sp.	Ascidian *Symplema* sp. Florida, USA	Zhang et al. (2016)
Ulbactins F & G	Anticancer	*Brevibacillus* TPB0800	Sponge, Coast of Japan	Igarashi et al. (2016)
Thiasporines A–C	Anticancer	*Actinomycetospora chlora* SNC-032	Sediment sample, Mangrove swamp, Vava'u Island, Tonga	Fu et al. (2015)
Mohangic acids A–E	Quinone reductase induction activity	*Streptomyces* sp.	Mudflat, Buan, Republic of Korea	Bae et al. (2016)
Naphterpin derivatives	Antioxidant	*Streptomyces* sp. CNQ-509	Sediment sample (La Jolla, CA, USA)	Park and Kwon (2018)

marine actinobacteria are given in Table 11.2. The members of the genus *Streptomyces* continue to be the leading source for novel drug discovery. Moreover, marine-derived antibiotics are more efficient at fighting infections because the terrestrial bacteria have not developed resistance against them (Saha et al. 2006).

Nowadays, it is becoming gradually more difficult to find new through routine screening approaches. However, it is not the end of an era but threshold of an endless frontier. Rare or uncommon actinobacteria can be isolated by applying selective isolation procedures to poorly studied habitats including extreme environments (Okoro et al. 2009). Novel taxa from such ecosystems may contain unique compounds as the evolution of secondary metabolism (Jensen et al. 1996).

Fig. 11.2 Mangrove plants in Vellar estuary, Parangipettai, Tamil Nadu

11.1.3 Mangrove Ecosystems

Mangroves are salt-tolerant plants distributed in subtropical and tropical intertidal regions of the world which are also termed as "mangrove ecosystem" (Fig. 11.2). About 181,000 km^2 (Jusoff 2013) or about 75% of the world's tropical and subtropical coastline area (Azman et al. 2015) is covered by mangrove swamps. The mangrove ecosystems are very productive but highly sensitive and fragile in nature. Moreover, these ecosystems also harbor other plant and animal and microbial species. Anthropological pressures and natural calamities greatly affect the mangrove environment. The significant physical features of the mangrove that assist their survival in the boundary zone between ocean and land are adaptations for mechanical fixation in loose soil, air exchange devices, breathing roots, specialized dispersal mechanisms, and specialized mechanisms for dealing with surplus salt concentration (Spalding 2010). Growing industrial areas beside the coastlines and discharge of domestic and industrial sewage are polluting this ecosystem. Experiences have confirmed that the presence of mangrove areas on coastline saves lives and property during natural calamities such as cyclones, storm surges, and erosion. These ecosystems are also well-known for their economic importance. Mangrove ecosystems act as grounds for feeding, breeding, and nursery for several marine organisms which made this ecosystem useful for captive and culture fisheries. The ecosystem has a very large unexplored potential for natural products used for medicinal purposes and also for salt production, fuel, fodder, apiculture, etc. The mangrove ecosystem is mainly an unexplored source of actinobacteria with the potential to synthesize active secondary metabolites (Hong et al. 2009). Even though several studies on bacterial efficiency and activity within the mangrove ecosystems were conducted, little is known about their hereditary and metabolic diversity. Both

the culturable and culture-independent studies revealed that mangroves harbor a great diversity of novel rare actinobacteria (Meena et al. 2013; Azman et al. 2015).

Globally, there are only limited studies that illustrated the diversity and isolation of novel rare actinobacteria in different mangrove territories. A rifamycin-producing *Micromonospora* from mangrove in the South China Sea was reported by Xie et al. (2006). The occurrence of *Micromonospora* from the Sunshine Coast in Australia was reported by Eccleston et al. (2008). Rare actinobacterial genera such as *Microbacterium*, *Rothia*, *Dermabacter*, *Kytococcus*, *Brevibacterium*, and *Nesterenkonia* were isolated from mangrove sediments in Brazil (Dias et al. 2009). *Actinomadura*, *Isoptericola*, *Microbispora*, *Nonomuraea*, *Rhodococcus*, and *Nocardia* were isolated from mangrove soils and plants in China (Hong et al. 2009). Results from Ara et al. (2013) illustrated that 17 rare actinobacteria genera were recognized from a total of 241 isolates. Notably, members of the genus *Micromonospora* were predominantly isolated from both mangrove and medicinal plant rhizosphere soil samples in Dhaka, Bangladesh. This result was in stripe with other studies (Jiang and Xu 1996; Hatano 1997; Ara et al. 2002) which revealed *Micromonospora* was the major genus from wet soil. Lee et al. (2014) reported that about 40.2% of actinobacteria isolated from Malaysian mangrove forest are rare actinobacteria, and in particular, the genus *Mycobacterium* was predominant. Other rare actinobacterial genera such as *Streptacidophilus*, *Leifsonia*, *Terrabacter*, and *Sinomonas* were also isolated from the same mangrove environment which are not generally reported from the mangrove environment. Notably, about 29 novel species of actinobacteria that come under 17 rare actinobacterial taxa are isolated from geographically different mangrove ecosystems in addition to several novel *Streptomyces* species during the years between 2001 and 2015. The isolated rare actinobacterial genera include *Agromyces*, *Asonoa*, *Polymorphospora*, *Nonomuraea*, *Micromonospora*, *Actinomadura*, *Verrucosispora*, *Demequina*, *Sphaerisporangium*, *Isoptericola*, *Jishengella*, *Microbispora*, *Lysinimicrobium*, *Actinoallumurus*, *Microbacterium*, *Mumia*, and *Sinomonas* (Azman et al. 2015).

11.1.4 Indian Mangrove Ecosystems

Mangroves in India occupy an area of 4921 km^2 accounting nearly 0.15% of the country's total geographical area (State of Forest Report 2017). Among the mangrove ecosystems in Indian coastlines, the Sundarban mangroves occupy a very large area followed by the Andaman-Nicobar Islands and Gulf of Kutch in Gujarat. The rest of the mangrove ecosystems is comparatively smaller. Although they are distributed in almost all maritime states, major formations refer to the Sundarbans of West Bengal (2097 km^2), Gulf of Kutch of Gujarat (1103 km^2), Andaman and Nicobar group of islands (604 km^2), Krishna and Godavari delta of Andhra Pradesh (352 km^2), Bhitarkanika and Mahanadi delta of Odisha (213 km^2), some parts of Maharashtra (186 km^2), and Pichavaram of Tamil Nadu (39 km^2). Approximately 59 species of mangroves belong to 41 genera, and 29 families are present in Indian mangrove ecosystems. Sundarbans alone accounts for almost half of the total mangroves in India. There are about, in the west coast ecosystem, 25 mangrove

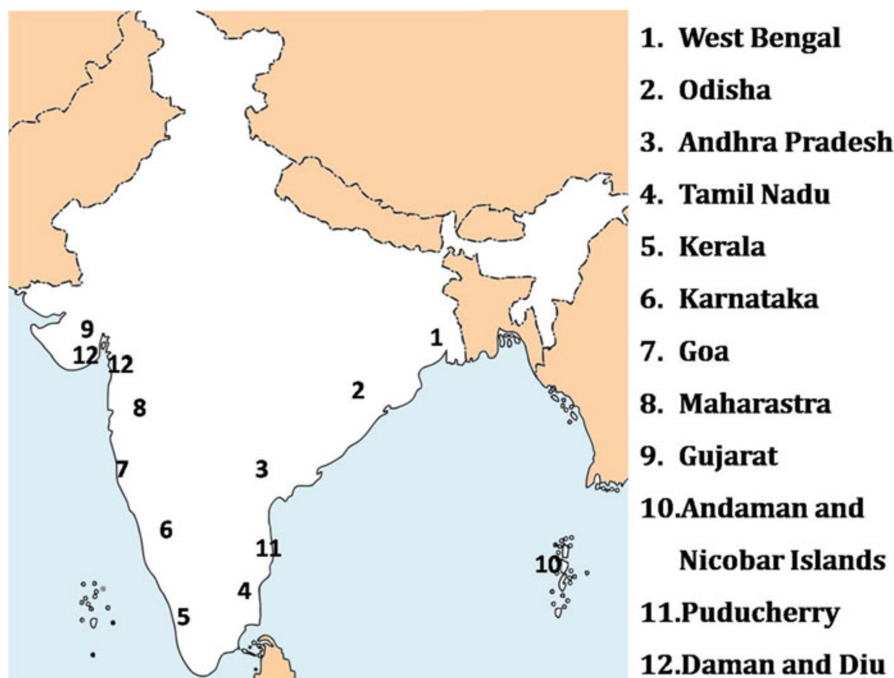

Fig. 11.3 Distribution of mangroves in different states of India

species that have restricted distribution along the east coast and are not found on the west coast. Similarly, mangroves like *Sonneratia caseolaris*, *Suaeda fruticosa*, and *Urochondra setulose* have been reported only from the west coast region. There are approximately 20 mangrove species reported from Maharashtra coastline followed by 16 species from Gujarat, 14 from Goa, and 10 from Karnataka. Along the Kerala coast, hardly three to four species of mangrove are reported. In general, *Rhizophora mucronata*, *Sonneratia* sp., *Avicennia* sp., *Excoecaria agallocha*, and *Acanthus ilicifolious* form the dominant mangrove plants of both east and west coast regions (Fig. 11.3).

11.1.5 Diversity of Actinobacteria in Indian Mangrove Ecosystems

The diversity and ecological and industrial significance of mangrove microbial communities have generated profound interest among microbial ecologists. The vibrant nature of the mangrove environment due to its regular tidal variations, pH, temperature, rainfall, salinity, light, and nutrient availability provides a remarkable, admirable environment for the enhanced growth of a wide range of microorganisms with diversified activities such as biogeochemical cycles and biocycling of most nutrients, including nitrogen (Imchen et al. 2017).

In 1988, Alongi described that in tropical mangroves, among the total microbial population, bacteria including actinobacteria and fungi occupy maximum of 91% of the total microbial population, followed by algae (7%) and protozoa (2%) (Alongi 1988). In the mangrove ecosystems, in general, the total actinobacterial population is dominated by *Streptomyces* followed by *Micromonospora*. More indication has shown that rising numbers of rare actinobacteria have been discovered from mangrove ecosystems.

11.1.6 Culture-Based Approach

Terrestrial ecosystems are well investigated for several decades in terms of actinobacterial diversity and bioprospecting potential, whereas studies on marine-derived actinobacteria are very less. Marine sediments are the richest source of actinobacteria from which several novel actinobacterial strains were isolated as well as their bioactive molecules were well explored (Goodfellow and Haynes 1984). Determination of diversity and distribution of actinobacteria are highly needed for their exploration.

Littoral zone and the deep sea are the two notable locations in the marine environment in which microbial population density including actinobacteria is higher in littoral sediments than the deep sea environment (Weyland 1969). Actinobacterial research has gained momentum in the last five decades starting from diversity, taxonomy, bioprospecting, and bioactive metabolites (Zobell 1946; Weyland 1969, 1981; Walker et al. 1975; Okami and Okazaki 1978; Jensen et al. 1991; Imada 2005; Imada et al. 2010). Baam et al. (1966) first initiated, in the Indian marine environment, the diversity of actinobacteria. Conditions like temperature, pH, salinity, moisture, and nutrient level in the mangrove ecosystem diverge greatly in different regions which greatly influence the diversity and taxonomic distribution of microbes including actinobacteria. Across the world, till date, more than 25 genera of actinobacteria belonging to different families and suborders were reported from mangrove ecosystems. Members of the actinobacterial genus *Streptomyces* have been predominantly distributed in all the mangrove ecosystems. Several studies on actinobacterial diversity in the mangrove ecosystems were conducted worldwide. However, in India, their study has been under-reported as scarce information is known about their bioprospecting application.

Research on actinobacteria predominantly from Indian mangrove ecosystem initially started from Tamil Nadu state in the early 1970s. In Tamil Nadu, mangroves are located on the Cauvery deltaic areas in notably intense mangrove forests with *Rhizophora* spp., *Avicennia marina*, *Excoecaria agallocha*, *Aegiceras corniculatum*, *Bruguiera cylindrica*, *Lumnitzera racemosa*, and *Ceriops decandra* present in Pichavaram mangrove ecosystem. In addition, mangroves also occur in near places like Vedaranyam, Kodiakarai (Point Calimere), Chatram, Muthupet, and Tuticorin. Even though the Pichavaram mangrove is very small in area, it has been very well investigated for ecology, biogeochemistry, widelife, microbial diversity, and bioprospecting by the researchers from several academic and research institutes.

Presence of the *Streptomycetes* species in the marine, estuarine, and mangrove sediments of Porto Novo was investigated by Paulraj in 1973. Later Lakshmana Perumalsamy (1978) identified five species of *Streptomycetes* community in the Pichavaram mangroves and isolated *S. olivacious*, *S. lucitanus*, *S. hydrogenus*, and *S. orientalis*. In 1979, Vanaja Kumar identified five species of *Streptomyces* from Pichavaram mangroves. These include *S. mutabilis*, *S. albosporius*, *S. halstedii*, *S. flavoviridis*, and *S. parvulus*. Balagurunathan (1992) identified *S. parvulus* and *S. alboniger* in the Pichavaram mangrove sediments. In the year 2005, Kathiresan et al. isolated 160 actinomycetes from the sediment samples collected from the mangrove rhizosphere region, estuarine, sand dune plants, and industrially polluted marine environments. The maximum number of isolates was obtained from coastal mangrove sediments (118 strains) and the minimum from industrially polluted coastal samples (5 strains). Remya and Vijaya Kumar (2008) isolated and characterized actinomycetes from west coast mangrove including 24 actinomycetes from the Calicut mangrove of Kozhikode Dt., Kerala, using SCA. One of the potential mangrove isolates was identified as *S. virodiviolaceus* (RM42). The occurrence and distribution of *Streptomyces* in the Bhitarkanika mangrove environment of Orissa was reported for the first time in the last decade (Gupta et al. 2009). They have isolated about 105 strains of *Streptomyces* belonging to 20 different species from the rhizosphere sediments collected from 19 different mangrove plants. The most prominent species isolated is *S. xanthochromogenes* followed by *S. exfoliates* and *S. auranticus*. Balagurunathan et al. (2010) isolated 20 strains of L-glutaminase-producing actinomycetes from mangrove rhizosphere sediments of Parangipettai coastal area, and all the isolates were identified as *Streptomyces olivochromogenes* P2. Arifuzzaman et al. (2010) isolated 55 strains belonging to four actinobacterial genera such as *Actinomyces*, *Nocardia*, *Streptomyces*, and *Micromonospora* from the sediment samples collected from the Karanjal region in Sundarban. Vijaya Kumar et al. (2010) studied the larvicidal potentiality of marine actinomycetes isolated from Muthupet mangroves, Tamil Nadu. A total of 30 actinomycetes were isolated from mangrove sediments using SCA supplemented with amphotericin B and penicillin. Twenty-three out of 30 isolates showed mosquito larvicidal activity, and in particular, 2 isolates CC11 (*Streptomyces* sp.) and SH23 (*Streptosporangium*) exhibited notable activity. Sathyaseelan and Stella (2011) isolated actinobacteria from Muthupet mangrove ecosystems using starch casein agar medium and obtained eight antagonistic actinomycetes in which one strain, namely, AP8, showed promising antibacterial, antifungal as well as α glucosidase inhibitor activity. Usha et al. (2011) isolated 63 actinomycetes from Pichavaram mangrove ecosystem, and they were screened for the production of L-asparaginase activity. In their study, asparagine, tryptone, dextrose, and NaCl were found to be the best medium components. Ravikumar et al. (2011) reported the biodiversity of actinomycetes along the southeast coast of India. The number of actinomycete isolates was maximum in the Karangadu mangrove region (62 strains) followed by Thondi (45 strains), particularly in the monsoon region. Sivakumar et al. (2005a) reported 91 strains of actinobacteria from different stations of Pichavaram mangrove ecosystem. Again, Sivakumar et al. (2005a, b) isolated actinobacteria from different

stations of Pichavaram mangroves using three different media. Consistently a higher number of actinobacterial populations were isolated on Kuster's agar, and the highest population density recorded was 4×10^4 CFU/mg. Sivakumar et al. (2005b) isolated antagonistic actinobacteria from Pichavaram mangrove ecosystem. Only one strain, identified as *Streptomyces roseolilacinus*, showed significant activity against bacterial and fungal pathogens. Ravikumar et al. (2011) reported the biodiversity of actinomycetes in Manakkudy mangrove ecosystems, south-west coast of India, using SCA supplemented with nalidixic acid, nystatin, and cycloheximide. In their study, the diversity of actinomycetes was found at maximum in the rhizosphere soil than in the non-rhizosphere soil at the depth of 10–20 cm, and even the nutrient level was found high at 0–10 cm. In addition, it was found that the mangrove associate *Acrostichum aureum* harbored maximum counts than true mangrove plants. Rajesh Kannan and Prakash Vincent (2011) investigated the molecular characterization of antagonistic *Streptomyces* sp. which were isolated from the sediment sample collected from the mangrove swamp of Manakkudy mangrove ecosystem located on the west coast of India using SCA supplemented with nalidixic acid. Their phylogenetic analysis revealed that all the 20 antagonistic isolates were having a strong similarity (98%) with *Streptomyces* which include 8 new species of *Streptomyces*. Govindasamy et al. (2011) isolated actinobacteria, in addition to other bacteria, for the first time from Muthukuda mangroves, southeast coast of India. Muthukuda mangroves cover a total area of 12 hectares where the mangrove *Avicennia marina* is the dominant species. Naikpatil and Rathod (2011) isolated actinobacteria from mangrove sediments of Karwar, Karnataka, using different isolation media and pretreatment procedures. The results revealed that untreated soil samples produced more bacterial colonies followed by *Streptomyces*, fungi, and rare actinobacteria, whereas the air-dried samples yielded an increased number of *Streptomyces* colonies. When the sediment sample was dried at 100 °C for 1 h, all the microbial population including rare actinobacteria were decreased. The recovery of rare actinobacterial population was increased up to 50% when the sample soil suspension was heated at 70 °C for 15 min. Likewise, phenol (1.5%) treatment of soil sample results in the enhanced recovery of rare actinobacteria. Hair hydrolysate vitamin agar (HHVA) was found to be suitable for the recovery of *Streptomyces* and rare actinobacteria. A total of 53 rare actinobacterial strains were identified coming from the genera *Micromonospora*, *Microbispora*, *Actinomadura*, and *Actinoplanes*, of which 13 strains showed antimicrobial activity. Patil et al. (2011) isolated seven different actinobacterial strains from the mangrove swamp collected from Achara, Malvan, Maharashtra, using glycerol asparagine agar and AIA. Based on the molecular characteristics, they were identified as *Cellulomonas* (MS1), *Corynebacterium xerosis* (MS2), *Streptomyces* sp. (MS3), *S. coelicolor* (MS4), *Streptomyces* sp. (MS5), *S. niger* (MS6), and *Streptomyces* sp. (MS7). Rajkumar et al. (2012) studied the actinobacterial diversity in the Bhitarkanika mangrove environment, east coast of Orissa. A total of 116 actinobacterial colonies were recovered from 30 mangrove and marine sediments using Kuster's agar. Of the 67 morphologically distinct isolates, 43 were assigned to the genus *Streptomyces*, 5 to *Actinopolyspora*, 5 to *Nocardiopsis*, 3 to *Micromonospora*, 5 to *Saccharopolyspora*, 5 to *Actinomadura*, and 1 actinomycete based on their

phenotypic and cell wall chemical characteristics. They also studied the physico-chemical properties of the samples. Priyanka Kishore isolated and characterized 15 actinobacterial isolates from Bhitarkanika mangrove ecosystem, Orissa. They were identified as the potential producer of various enzymes and for antimicrobial properties. Ragava Rao et al. (2012) had isolated 20 actinobacteria from the mangrove region in Visakhapatnam, Andhra Pradesh, using SCA, glucose–YEME agar, and Actinomycetes isolation agar. All the isolates were identified as *Streptomyces* sp. Rare actinomycetes from the mangrove ecosystem of Nizampatnam, Andhra Pradesh, were isolated using glycerol-asparagine agar, and they were identified based on the polyphasic taxonomic studies. Strain VUK10, which showed good antimicrobial activity, was identified as *Pseudonocardia.* Nithya et al. (2012) isolated and characterized actinomycetes from Manakkudy mangrove ecosystem as well as from Yercaud Hills and also studied their antibacterial and anticancer activities. In this study, 130 actinomycete strains were isolated from the mangrove region, and they were found to be superior in overall antibacterial activity when compared to the strains picked up from the Yercaud Hills. Nag et al. (2012) isolated actinomycetes from the rhizosphere sediments of Valapattanam and Pappinisseri mangroves located in the Kannur District, Kerala, using starch casein agar (SCA), and some potential actinomycetes were identified as *Streptomyces orientalis* (A10), *S. viridodiasaticus* (A11), *S. antibioticus* (A16), *S. albus* (A18), *Micromonospora* (A21), *S. badius* (A23), *S. lavendulae* (C14), and *S. aureomonopodiales* (C15) based on their phenotypic characteristics. Sweetline et al. (2012) isolated 38 actinomycete strains from 4 soil samples collected from Pichavaram mangrove ecosystem, Tamil Nadu, using SCA medium. Seventeen out of 38 isolates were found to be antibacterial producers by cross streak method. Twenty-seven isolates of *Streptomyces* were recovered from Coringa mangrove forest using ISP2 agar. Further, a maximum number of isolates showed various enzymatic activities like amylase, protease, cellulase, chitinase, and L-asparaginase. Actinobacteria from the mangrove sediment samples collected from Andaman Nicobar Islands were isolated using different pretreatment methods and isolation media (Baskaran et al. (2011). Maximum actinobacterial density was obtained in Kuster's agar followed by Actinomycetes isolation agar and starch casein nitrate agar medium. In another study, phyllosphere samples were collected from ten different mangrove species in the Andaman Islands and were investigated for the actinobacterial as well as other bacterial and fungal diversity. The results revealed that the occurrence of the bacterial, fungal, and actinobacterial populations was uniform at uniform salinity. It was also noted that maximum actinobacterial population was obtained from the samples collected from *B. gymnorhiza* using Actinomycetes isolation agar.

Janaki et al. (2014) had isolated about 100 actinobacterial strains including 14 anaerobes from the Pondicherry mangrove regions using different sediment treatment and isolation media. Actinobacterial diversity in the Palk Bay region was investigated by collecting sediment samples at four different seasons. Of the total 57 actinobacterial strains, six strains were predominantly isolated in all the four seasons. The six isolates were identified as *Streptomyces niveoruber*, *S. heliomycini*, *S. flavomacrosporus*, *Lechevalieria aerocolonigenes*, *L. flava*, and *Dactylosporancium vinaceum* based on their phenotypic and molecular

characteristics (Priya et al. 2014). Fifty-four actinomycetes were isolated by Sengupta et al. (2015) from an unexplored region of Sundarban mangrove ecosystem and their antagonistic activity reported against the battery of 15 test pathogens including three phytofungal pathogens. Palla et al. (2018) isolated actinomycetes from sediment samples collected from Coringa mangrove forest in Kakinada, Andhra Pradesh, using starch casein nitrate agar medium prepared using 50% seawater.

Sivakumar et al. (2007) affirmed that till 2007, about 41 species of actinobacteria belonging to 8 genera have been reported from the Indian mangrove ecosystem. Majority of the studies were carried out mainly at mangroves of four states, namely, Tamil Nadu, Kerala, Andhra Pradesh, and Andaman and Nicobar Islands. The genus *Streptomyces* was most predominantly isolated from the mangrove regions in India. But, in the last decade, there are more reports on rare actinobacteria from Indian mangrove ecosystems (Table 11.3). Most of them are reported from mangrove rhizosphere sediments. At least 22 rare actinobacterial genera were reported from Indian mangrove ecosystems.

Endophytes, the microbes living within the healthy plant tissues without causing ill effects, are recognized as a tremendous source for novel bioactive metabolites with clinical, environmental, and agricultural applications. As like other ecosystems, different actinobacteria notably *Streptomyces* are predominantly present in several plants as endophytes with the potential to produce several bioactive metabolites and enzymes. Several novel metabolites with different indications like antibacterial, antifungal, antitumor, antiviral, and immunomodulatory properties were reported from endophytic actinobacteria.

In several studies, the genus *Streptomyces* dominated the total endophytic actinobacterial populations recovered from different parts of medicinal plants. Other rare actinobacterial genera, viz., *Micromonospora*, *Nocardia*, *Actinopolyspora*, *Streptosporangium*, *Saccharopolyspora*, *Promicromonospora*, and *Rhodococcus*, were also isolated as endophytes. In India, there are very few studies on endophytic actinobacteria from mangrove plants. Ravikumar et al. (2011) isolated 17 strains of endophytic actinobacteria from the leaves of 5 mangroves and mangrove associates such as *Avicennia marina*, *Bruguiera cylindrica*, *Rhizophora mucronata*, *Salicornia brachiata*, and *Suaeda monoica* collected from the Karangkadu mangrove ecosystem in Tamil Nadu. In the preliminary antimicrobial screening, 10 out of 17 strains inhibited the test pathogens. In our lab, an endophytic actinobacterium, identified as *Streptomyces* SACC4, was isolated from the leaves of *Rhizophora apiculata* collected from Parangipettai mangrove ecosystem. Crude bioactive extracts of the strain SACC4 showed promising antibacterial and antitubercular activity (unpublished data).

11.1.7 Metagenomic Approach

Molecular phylogenic studies have exposed that only a small number (<1%) of microbial diversity present across the globe are readily cultivable using conventional

Table 11.3 List of rare actinobacteria from Indian mangrove ecosystem

Rare actinobacterial genera	Sample	Ecosystem	References
Micromonospora, *Microbispora*, *Actinoplanes*, *Actinomadura*	Sediment	Mangroves forest of Karwar	Naikpatil (2014)
Actinomadura sediminis	Sediment	Dugong Creek, little Andaman, India	Xu et al. (2012)
Pseudonocardia endophytica	Sediment	Nizampatnam mangrove ecosystem	Mangamuri et al. (2012)
Actinopolyspora, *Actinomadura*, *Nocardiopsis*, *Micromonospora*	Sediment	Vellapallam, and around Vedaranyam, near Nagapattinam, Tamil Nadu	Deepa et al. (2013)
Thermoactinomyces	Sediment	Velapallam mangrove, Tamil Nadu	Deepa et al. (2013)
Saccharopolyspora, *Nocardiopsis*, *Streptoverticillium*, *Microtetraspora*, *Actinopolyspora*, *Actinokineospora*, and *Dactylosporangium*	Sediment	Andaman and Nicobar Islands	Meena et al. (2013)
Actinomadura, *Actinokineospora*, *Catellospora*, *Kitasatosporia*, *Nocardia*, *Nocardiopsis*, *Planobispora*, *Planomospora*, *Terrabacter*, *Saccharothrix*, *Streptosporangium*, *Streptoverticillium*	Sediment	Palaverkadu mangrove, Tamil Nadu	Karthikeyan et al. (2014)
Lechevalieria aerocolonies, *Dactylosporangiun vinaceum*	Sediment samples	Muthupet mangrove ecosystem	Priya et al. (2014)
Saccharomonospora oceani VJDS-3	Sediment samples	Nizampatnam mangrove ecosystem	Indupalli et al. (2018)
Arthrobacter	Sediment samples	Bhitarkanika mangrove forest, Odisha	Satapathy and Mohapatra (2017)

culture-based approaches (Yan et al. 2006). It is apparent that, as in the case of bacteria, four phyla, namely, Proteobacteria, Actinobacteria, Firmicutes, and Bacterioidetes, occupied more than 88% of the bacterial population (Nikolaki and Tsiamis 2013). Metagenomics is a culture-independent genome analytical approach to determine the microbial diversity in any given sample. This modern approach is reducing difficulties related to circumstances for bacterial cultivation. As >98% of microbes are not yet cultivable by adopting routine culture-based approaches, metaganomics is the prime option for the analysis of microbial diversity for further exploration. Until now, the few metagenomic studies in mangroves have been concentrated only in South America. The South American mangrove ecosystems are important but constitute only 11% of the range of mangroves across the globe. In particular, there are no in-depth studies on the diversity of actinobacteria from Indian mangrove ecosystems, especially through metagenomic approach. In 2017, Imchen et al. (2017), for the first time, carried out the detailed metagenomic analysis of four

different mangrove locations across India. They also compared the microbial diversity of the studied Indian mangrove ecosystem versus a similar Brazilian mangrove ecosystem.

Basak et al. (2015) for the first time reported the microbial community present in the mangrove present in Dhulibhashani, Sundarbans, using 16S rRNA tag sequencing. The sediments were collected inside the mangrove during monsoon and postmonsoon season in the year 2013. Among the total microbial community, the actinobacteria accounted for 7% for the post-monsoon at two different depth samples (2 cm, Dec 2013) and only 5% for the monsoon sample only in the surface sample (2 cm, July 2013). Pramanik et al. (2016) studied the bacterial community structure at Paradip Port, Odisha, India, through the metagenomic approach. The results revealed that the marine sediment sample contains 7% of actinobacteria among the total bacterial diversity.

11.1.8 Role of Actinobacteria in Mangrove Environment

Actinobacteria are prominently distributed in the soil where they play a vital role of biogeochemical cycling of various complex carbohydrates like starch, cellulose, chitin, pectin, xylan, etc. They can also be able to degrade some recalcitrant molecules like pesticides (Goodfellow and Haynes 1984). They are responsible for the pleasant odor of freshly turned soil due to their production of the volatile compound, geosmin (Mincer et al. 2002). In addition, they play a significant role in the mineralization of organic matter, immobilization of mineral nutrients, fixation of nitrogen, environmental protection, and improvement of physicochemical conditions (Goodfellow and Williams 1983). Actinobacteria have a profound role in the marine environment apart from the production of bioactive metabolites (Das et al. 2006). The biogeochemical cycling of various complex substrates into simple forms in any ecosystem is mediated by the enzymatic potential of microorganisms. Microbes thereby increase the nutrient level and productivity of such ecosystems (Jensen et al. 2005; Lam 2006). Inversely, the population density of a particular group of enzyme-producing microorganisms in an ecosystem is an indication for the fluctuation in nutrient levels in such ecosystems (Ramesh and Mathivanan 2009). Actinobacteria are able to produce several enzymes to degrade complex substrates which make them as ubiquitous organisms in any normal and extreme environments. The cellulolytic activity of marine actinomycetes was described by Chandramohan et al. (1972). Production of enzymes with industrial, clinical, medical, and environmental importance such as protease, amylase, lipase, pectinase, cellulose, chitinase, L-asparaginase, and L-glutaminase was reported by several authors (Pisano et al. 1992; Ramesh and Mathivanan 2009; Balagurunathan et al. 2010).

Microbial enzymatic activities in the decomposition of mangrove leaves of *Rhizophora mucronata* Poir. (Rhizophoraceae) and *Avicennia marina* Forsk. (Vierh) (Avicenniaceae) of the Vellar estuary in the southeast coast of India (Lat. 26°49′N; long. 46°96′E) were studied. The results showed that the actinobacterial counts increased with decomposing leaves, reached a peak between 32 and 40 days

of decomposition, and declined thereafter. In *Rhizophora mucronata*, the actinobacteria ranged from 0.001×10^8 to 0.364×10^8 CFU.g^{-1} in the leaves decomposed for 8 and 40 days, respectively. In *Avicennia marina*, the counts ranged from 0.001×10^8 to 0.426×10^8 CFU.g^{-1} in the leaves decomposed for 8 and 40 days, respectively. Actinobacterial counts between days were significant, but not between the mangrove species (Kathiresan et al. 2011).

11.1.9 Bioprospecting Potential of Indian Mangrove Actinobacteria

Members of the phylum actinobacteria are very unique in their metabolic and biosynthetic potential due to the presence of a vast array of secondary metabolic gene clusters which are not commonly present in other organisms. There are several high-value secondary metabolites including antibiotics and anticancer agents that are isolated from different actinobacterial genera, notably *Streptomyces* (Balagurunathan and Radhakrishnan 2010; Berdy 2012). But the bioprospecting potential of actinobacteria from Indian mangrove ecosystems has been under-investigated and under-reported.

11.1.10 Primary Metabolites

Primary metabolites are produced by microorganisms during the active growth phase (exponential/trophophase) where the essential nutrients or substrates are present in the growth medium. Primary metabolites are produced by almost all the microorganisms since they are essential for their growth and multiplication. Primary metabolites are either the end and intermediate products of anabolic reaction like nucleotides and amino acids, or they are transformed into co-enzymes like vitamins. Other primary metabolites (e.g., acetic acid, citric acid, and ethanol) result from catabolic reactions which are not used for building cellular constituents but their production is associated with energy production, and substrate utilization is necessary for growth (Sanchez and Demain 2008).

Next to antibiotics, enzymes are the important products produced by actinobacteria. Enzymes are the major primary metabolites produced from Indian mangrove actinobacteria, and they are not much investigated for other primary metabolites. Twenty-seven actinobacterial strains isolated from Coringa mangrove forest were screened for various extracellular enzymatic activities. *Streptomyces* sp. strain A showed a wide range of enzymatic activities such as amylase, cellulose, asparaginase, chitinase, and protease (Kavya Deepthi et al. 2012). Actinobacterial strains isolated from Muthupet mangrove ecosystem were investigated for extracellular enzyme production. About 35 out of 205 actinobacterial strains exhibited amylase (25%), protease (21%), lipase (16%), esterase (17%), and gelatinase (21%) activity (Sathya and Umadevi 2014). About 6 out of 31 actinobacterial strains isolated from Nizampatnam mangrove ecosystem were found to produce L-asparaginase enzyme. The potential strain *Pseudonocardia endophytica* FM-4

was found to produce 3.96 IU/ml of L-asparaginase in submerged fermentation (Usha Kiranmayi et al. 2014). Twenty-six actinobacterial strains belonging to eight different genera were isolated from Minnie Bay of Andaman and Nicobar Islands and were screened for various extracellular enzymes such as amylase, gelatinase, lipase, protease, DNase, cellulase, urease, and phosphatase. About 77% of the strains showed significant hemolytic activities (Meena et al. 2013). In another study, 14 out of 20 actinobacterial strains isolated from the mangrove coastal area of Maharashtra-Goa border were found to exhibit cellulolytic activity. Notably, two strains Ac1 and Ac3 identified as *Streptomyces* sp. were found to produce maximum cellulose on a different substrate.

11.1.11 Secondary Metabolites

Secondary metabolites are exclusively produced by selected microorganisms which are not vital for the growth and reproduction of those producing organisms. In general, filamentous microbes like molds and filamentous members of the phylum *Actinobacteria* are reported to have diverse secondary metabolite biosynthetic genes. The nutritional components like carbon source, nitrogen source, minerals, and culture conditions like pH and temperature greatly influence the secondary metabolite production. When compared to primary metabolite production, the regulation of secondary metabolite production is very complex. Unlike primary metabolite production, secondary metabolites are produced at the end of the stationary phase, i.e., idiophase. Even though the secondary metabolites are not essential for the growth and reproduction of producing microbes, they are produced abundantly by certain filamentous microbes like *Streptomyces*. Microbial secondary metabolites are exhibiting a wide range of biological activities such as antibiotic, anticancer, antiviral, and immunomodulatory properties (Berdy 2012). *Actinobacteria* are well explored for several novel secondary metabolites, notably antibiotics, but it is meager in the Indian context. Here we described the reports on antimicrobial activity of actinobacteria isolated from Indian mangrove ecosystems. Remya and Vijayakumar (2008) investigated the distribution and antimicrobial activity of actinobacteria isolated from sediment samples collected from eight different locations of Kerala, west coast of India. Out of 64 isolates, 21 had antimicrobial activity, and in particular, two strains showed broad-spectrum activity. A bioactive secondary metabolite, 2-allyloxyphenol, exhibiting anticancer and antimicrobial activities was produced from marine actinobacterial strains MS1/7(T), isolated from sediments of the Sundarban mangrove forest, India (Arumugam et al. 2017). Kannan and Vincent (2011) had studied the antimicrobial potency of the actinobacteria isolated from rhizosphere soil of Manakkudy mangroves, west coast of India. In their study, all the 20 isolates showed antagonistic activities against various bacterial and fungal pathogens. Naikpatil and Rathod (2011) had isolated 53 strains of rare actinomycetes from the mangrove sediments of Karwar coast using different pretreatment methods as well as different media. Two rare actinobacterial strains, viz., *Actinomadura* sp. and *Micromonospora* sp., demonstrated promising

antimicrobial activity with MIC values of 5 mg/ml and 1.2 mg/ml, respectively. Pichavaram mangrove ecosystem is well investigated for actinobacteria when compared to other mangrove regions in Tamil Nadu. Sweetline et al. (2012) isolated and studied the antimicrobial activity of 38 actinobacterial strains recovered from the sediment samples collected from Pichavaram mangrove ecosystem. In the cross streak method, 17 out of 38 actinobacterial strains were found to be active against the tested bacterial pathogens. In particular, the strain KMA02, identified as *Streptomyces* sp., showed the maximum activity against all pathogens tested. Actinobacteria from the sediment samples collected from Coringa mangrove forest, Andhra Pradesh, were isolated by adopting dilution-plate technique (Deepthi et al. 2012). Among the 27 actinobacterial strains isolated and screened for enzymatic and antimicrobial activities, one *Streptomyces* sp. A1 showed enzymatic activities of amylase, chitinase, cellulase, L-asparaginase, and protease as well as antimicrobial activity against *S. aureus*, *P. aeruginosa*, *P. fluorescence*, *L. acidophilus*, *L. casei*, *C. albicans*, *S. mutans*, *B. subtilis*, *B. megaterium*, and *Xanthomonas* sp. Maximum activity was exhibited by *Streptomyces* sp. A1 against *Xanthomonas*, whereas minimum was exhibited against *Candida albicans*. Mangamuri et al. (2012) isolated actinobacteria from the sediments collected from Nizampatnam mangrove ecosystem and reported their antimicrobial activities. Similarly, actinobacteria from the mangrove sediments were collected from the mangrove zone of Maravakkadu Reserve Forest, Thanjavur district, Tamil Nadu. Three species showed antimicrobial activity against both Gram-positive and Gram-negative bacterial pathogens (Ashok et al. 2014). GC-MS analysis of methanol extract obtained from *Streptomyces albus* revealed the presence of seven compounds. The peak indicates that the compounds were determined as 1-octanol, 2,7-dimethyl (8.56Rt, oxalic acid, allylnonyl ester (12.79Rt), pentanoic acid, 4-methyl (17.81Rt), n-hexadecanoic acid (18.90Rt), oleic acid (22.14Rt), 1,10-hexadecanediol (29.56Rt), and griseofulvin (33.93Rt). Mangamuri et al. (2014) compared the spatial distribution of actinobacteria and the physicochemical properties of mangrove sediments of Nizampatnam and Coringa located along the southeast coast of Andhra Pradesh, India. In antimicrobial screening, 28 out of 58 strains were found to exhibit antagonistic activity. Further, they were also reported to produce some extracellular enzymes such as L-asparaginase, cellulase, and amylase. Das et al. (2014) isolated 25 alkaliphilic and halotolerant actinobacterial strains from a mangrove reported that actinobacteria from the sediment samples of Valapattanam mangrove swamp in Kerala, India. In the preliminary antimicrobial activity screening against nosocomial pathogens by well diffusion method, isolate no I-1 significantly inhibited a wide range of Gram-positive bacteria like *S. aureus* ATCC 25923, *S. aureus*, *S. citrus*, *B. cereus*, and *Serratia marcescens* with 12–20 mm zone of inhibition. It also demonstrated effective antifungal action against *Penicillium* sp., *Candida albicans*, *C. parapsilosis*, and *C. neoformans* with zone of inhibition between 12 and 20 mm. Sengupta et al. (2015) isolated 54 morphologically different actinobacterial strains from Sundarbans mangrove ecosystem and analyzed their antimicrobial potential toward a battery of microorganisms including three phytofungal pathogens. Among the nine actinobacterial strains, three specific strains were found to have

notably higher degree of antimicrobial potential effective in a broader range including antiphytofungal activity. One potential strain, SMS_SU21, showed antimicrobial activity with an MIC value of 0.05 mg ml^{-1} and antioxidant activity with an IC50 value of 0.242 ± 0.33 mg ml^{-1}. True prospect of this strain was evaluated utilizing GC-MS, and the bioactive compound responsible for antimicrobial activity was identified. Mangamuri et al. (2015) isolated and identified *Pseudonocardia endophytica* VUK-10 from Nizampatnam mangrove ecosystem, Andhra Pradesh, using glucose asparagine agar. In agar plate diffusion assay, bioactive extracts from this actinobacterium showed broad-spectrum activity against a wide variety of Gram-positive and Gram-negative bacteria and fungi. The anticancer activity was tested by MTT assay, and the compounds from this strain showed activity against four cancer cell lines, viz., human breast adenocarcinoma cell line (MDA-MB-231), human cervical cancer cell line (HeLa), human ovarian cyst-adenocarcinoma cell line (OAW-42), and human breast adenocarcinoma cell line (MCF-7) (cell lines reported to be resistant to cancer drugs). Compound PE-V1 purified from *Pseudonocardia endophytica* was found to be active against *S. aureus*, followed by *S. mutans*, *S. epidermidis*, *B. megaterium*, and *B. cereus*. Compound PE-V2 presented the highest activity against *B. cereus*, followed by *S. mutans* and *S. epidermis*. Gopalakrishnan et al. (2016) have isolated 23 actinobacterial colonies from the mangroves of Andaman and Nicobar Islands, India. Among the actinobacteria *Micromonospora* sp., *Streptomyces aburaviensis*, *Streptomyces aurantiogriesius*, *Streptomyces aurofasciculus*, *Streptomyces flavoviridis*, and *Nocardia astreoides*, *S. aurantiogriesius* has shown the maximum activity with more than 10 mm zone of inhibition against *Klebsiella* sp., *E. coli*, *V. cholerae*, and *S. flexheri*, whereas *S. aburaviensis* has shown the highest activity against *Klebsiella* sp., *E. coli*, and *V. cholerae*. The other *Streptomyces* species like *S. aurofasciculus* and *S. flavoviridis* have shown moderate activity against the pathogens. Janaki et al. (2016) have isolated 25 aerobic mangrove actinomycetes from the soil collected near the root region of *Avicennia marina* (Forsk) Vierh-(Avicenniaceae), Ariyankuppam backwater area. Out of 25 strains, 13 actinomycetes (52%) showed anti-candidal activity. The most active isolate was identified as *Streptomyces cacaoi* subsp. cacaoi (GenBank accession no.: KP872910); 100 μl of culture filtrate showed maximum inhibitory zone measuring as 28 mm in the well diffusion method. Kalpana Devi and Usha (2017) made a study focusing on the isolation of fascinating actinomycetes from the mangrove region. One hundred and ten actinomycetes were isolated from mangrove soil sediments from Pichavaram. Due to the broad spectral activity, the study was emphasized on the 54 strains that possess good activity. The antagonistic activities of the chosen actinomycetes were tested. The isolates were streaked on agar plate, and then the test pathogens (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Escherichia coli*, *Enterococcus faecalis*, and *Serratia marcescens*) were cross streaked. *Nocardiopsis prasina* ACT 24 showed very good activity against the selected human pathogen vancomycin-resistant *Enterococcus*. The screening reveals that antibiotic from ACT24 has potential to inhibit the vancomycin-resistant enterococci, and this can be exploited for further treatment in the future by analyzing the structure of the compound. Kiran et al. (2017) have isolated and investigated the antibacterial and

amylase enzyme-producing potential of actinomycetes from mangrove sediments collected from the Krishna estuary, Machilipatnam, Andhra Pradesh. One potential strain M-2 showed antibacterial activity with maximum inhibition of 27 mm against *Enterobacter aerogenes* followed by 32 mm against *Bacillus subtilis*, and it also produced 4.2 U/mL of amylase enzyme. Endophytic actinobacteria were isolated from two mangrove plants, *Rhizophora mucronata* and *Sonneratia caseolaris*, collected from Cortalim, Goa, by adopting a three-step sample pretreatment protocol and serial dilution cup standard spread plate method. Among the 11 strains isolated and tested for antimicrobial activity by the cross streak method and agar well diffusion method, the ethyl acetate extract of strains RO7 and RO11 showed promising broad-spectrum antibacterial activity, whereas the strain RO9 was found to be active against bacterial and fungal pathogens. In addition, the ethyl acetate extract RO7 showed maximum 94.11% radical scavenging activity in DPPH antioxidant assay (Mesta 2017). In another study, Indupalli et al. (2018) isolated a rare actinobacterial species from the mangrove sediments collected from Nizampatnam, Andhra Pradesh, India, and screened for its bioactive metabolite production. Based on the poolyphasic taxonomy, the potential strains were identified as *Saccharomonospora oceani* VJDS-3. Three bioactive metabolites, viz., methoxyethyl cinnamate (ethyl(E)-3-(4- methoxyphenyl)acrylate) (R1), 4-hydroxy methyl cinnamate (methyl(E)-3-(4-hydroxyphenyl) acrylate) (R2), and 4-methylbenzoic acid (R3), were isolated through bioassay-guided fractionation and identified through their physicochemical properties. They also reported the antimicrobial, antioxidant, antidiabetic, and anti-obesity properties of the isolated metabolites. This was the first report on the isolation of these potential compounds from the genus *Saccharomonospora*. Actinobacteria from mangrove sediments of Muthupet mangroves and Andaman and Nicobar Islands were assessed for their antifungal potential against 16 clinical strains of *C. albicans*. The 16 s rRNA analysis revealed that majority of the actinobacterial strains belonged to the genus *Streptomyces*. The PCR-based screening of biosynthetic genes revealed that maximum of 11 strains have the NRPS genes, whereas PKSI and PKS II genes were present in 6 and 4 actinobacterial strains, respectively. Two actinobacterial strains VITGAP240 and VITGAP241 showed promising antifungal activity. Extracts from both the strains contained the heterocyclic compounds, polyketides and peptides. Sengupta et al. (2018) studied the antimicrobial and antioxidant potential of *Streptomyces* sp. SMS_SU21 isolated from the Sundarbans mangrove ecosystem. They also described its draft genome comprising 7,449,420 bp with 6680 open reading frames which revealed the secondary metabolite potential of the strain SMS_SU21. Antagonistic actinobacteria from mangrove sediments were collected from the Coringa region, Kakinada, Andhra Pradesh, by Palla et al. (2018) and were studied for their antagonistic activity against various bacteria and fungi. One potential strain, KMFA-1, identified as *Streptomyces hydrogenans*, showed prominent activity against dermatophytes such as *Candida albicans* and *Pectinotrichum llanense*.

Although different types of anti-TB agents are available in the world market, there is a growing interest in natural products for novel anti-TB drug discovery, due to nonspecific side effects associated with synthetic therapeutic agents and unusual chemical diversity present in natural products. Marine-derived actinobacteria are the

promising source of anti-TB metabolites. Radhakrishnan et al. (2010) screened crude bioactive compounds from 15 actinobacterial strains isolated from rare marine and forest ecosystems for antitubercular activity by adopting luciferase reporter phage (LRP) assay. The actinobacterial strains D10, D5, NEK5, ANS2, M104, and R2 showed prominent activity, in which *Streptomyces* sp. ANS2 isolated from Andaman mangrove sediments and the *Micromonospora* strain M104 isolated from Parangipettai mangrove region, Tamilnadu, showed promising activity against the standard and clinical strains of *M. tuberculosis*.

Antimycobacterial activity of 49 actinobacterial strains isolated from the sediment sample collected from the mangrove area of Pichavaram, Parangipettai, and Andaman and Nicobar Islands was investigated (Radhakrishnan et al. 2011). Cell-free supernatant and mycelia methanol extracts of 30 *Streptomyces* and 19 rare actinobacteria which include 11 *Micromonospora* strains were screened for anti-TB activity against the standard strain *M. tuberculosis* H37Rv and clinical drug-sensitive and MDR strains of *M. tuberculosis* by adopting luciferase reporter phage (LRP) assay. Interestingly, culture filtrate and/or mycelia methanol extracts from 41 out of 49 actinobacterial strains inhibited at least one of the *M. tuberculosis* strains tested. Culture filtrates from actinobacterial strains, viz., R6 (*Streptomyces* sp.), M1A6 (*Micromonospora* sp.), M1A15 (*Actinosynnema* sp.), M1A18 (*Micromonospora* sp.), and M1A23 (Rare actinobacteria), showed more than 90% reduction in RLU by LRP assay. These studies evidenced that mangrove-derived actinobacteria are the promising source for novel anti-TB metabolites.

11.1.12 Aquaculture Probiotic Potential of Actinobacteria

The whole microbial cells have also been used as a product with clinical, environmental, agricultural, and aquaculture applications. This includes biofertilizer, probiotic, biocontrol agent, and single-cell proteins. Certain studies reported the probiotic potential of marine actinobacteria in India. Selvakumar and Dhevendaran (2014) studied the single-cell protein potential of selected sponge-associated *Streptomyces* on the growth of *Xiphoporus helleri.* After 30 days of feeding trials, growth parameters such as absolute growth rate, specific growth rate, and feed conversion efficiency were found to be significantly ($P < 0.001$) higher in groups that received *Streptomyces* as SCP feed as compared to control group. Thus, it was found that in addition to being effective antibiotic agents against harmful pathogens, *Streptomyces* could also promote the growth of fish effectively. In another study, probiotic potential of marine *Streptomyces* in laboratory culture of *Penaeus monodon* was evaluated by Das et al. (2006). The results showed that, when compared to control, the experimental culture tanks provided with *Streptomyces* had better water quality parameters. Further, it was also noted that the growth also increased as the *Streptomyces* cell concentration increased. Growth in length and weight was 15.79% and 57.97%, respectively, at 10 grams concentration, and the interval between molts was 12.5 days.

11.1.13 Bioremediation Potential

Microbial bioremediation serves as an alternative and effective strategy to remove toxic contaminants from a polluted environment. Bioremediation of the contaminated sites employing indigenous microbes is highly advantageous as it is ideally adapted to the environmental conditions prevailing at the site to be remediated (Pushpanathan et al. 2014). Duddu and Guntuku (2015) screened about 27 actinobacterial strains isolated from Coringa mangrove sediments, Andhra Pradesh, for degradation of low-density polyethylene. One potential strain M4 showed significant reduction in weight of LDPE films after 4 weeks of incubation. The strain M4 was also able to grow in other complex substrates such as polyvinyl acetate, polycaprolactone, polyethylene oxide, and polyethylene glycol. Actinomycetes isolated from the same mangrove ecosystem were found to degrade 60% of phenol under laboratory conditions (Rani and Mani Kumar 2017). The ligninolytic potential of mangrove-derived actinobacteria was investigated by Niladevi and Prema (2005). A potential strain *Streptomyces psamoticus* NJP49 was reported to produce all three major ligninolytic enzymes such as manganese peroxidase, lignin peroxidase, and laccase with maximum activity at pH 7 and at a temperature of 30 °C. A *Streptomyces* strain isolated from Muthupet mangrove sediments, Tamil Nadu, was found to degrade polythene and plastics with a maximum degradation of 11.01 ± 0.41 μg and 12.04 ± 0.33 μg, respectively, on the 55th day of incubation (Kannahi and Sudha 2013). Another study reported that *Streptomyces* sp. A1 isolated from mangrove sediments in Tamil Nadu was reported to degrade pollutants from paper mill effluents. Overall, the *Streptomyces* sp. strain A1 removed well the inorganic pollutants and odor from both sterilized and unsterilized effluents (Sathya and Ushadevi 2017). About 35 strains of *Streptomyces* isolated from the rhizosphere sediment were collected from the mangrove *Avicennia marina* and were screened for degradation of Azo dye. One potential strain M20 was found to accumulate and degrade efficiently at a maximum of 81 mg/100 ml concentration in 7 days.

11.1.14 Antifouling Potentials of Indian Mangrove Actinobacteria

The problems associated with environmental biofouling and the limitation of currently available antifouling compounds have intensified the search for novel eco-friendly antifouling compounds from new sources. Some studies have found that bioactive compounds of mangrove actinobacteria possess a wide range of antifouling activities. Fifty-five bacterial isolates were recovered from fouling samples collected from Muttom, Parangipettai, Nagapattinam, and Ennore coastal areas. Morphologically distinct isolates were phenotypically characterized. The isolates belonged to species of the genera *Bacillus*, *Aeromonas*, *Micrococcus*, *Alcaligenes*, *Lactobacillus*, *Staphylococcus*, *Pseudomonas*, and *Kurthia*. Biofilm forming capacity of all the isolates was evaluated by adopting both plate and tube methods. Out of 55 bacterial isolates, 25 isolates produced positive results for

biofilm formation, where the species belonged to the genera *Staphylococcus*, *Micrococcus*, *Vibrio*, and *Alcaligenes* and were identified as strong biofilm producers. For the isolation of potential antifouling compounds, a total of 50 actinobacterial isolates were recovered from mangrove and estuarine sediment samples collected from Parangipettai and Pichavaram coastal areas, Tamil Nadu, and were phenotypically characterized. In the agar plug method, 42 out of 50 actinobacterial isolates inhibited a greater number of biofouling bacteria tested. Two actinobacterial isolates, viz., strain PM33 and strain PE7, showed promising activity against a maximum number of biofouling bacteria tested. Isolation of bioactive metabolites from both the actinobacterial strains will lead to the development of promising antifouling candidates (Gopikrishnan et al. 2013). In their study, an attempt has been made to screen and evaluate the antifouling potentials of the actinobacterial strain *Streptomyces fradiae* RMS-MSU isolated from the manakkudy mangroves of Tamil Nadu. Screening results showed that the ethyl acetate extract of *S. fradiae* RMS-MSU displayed a wide spectrum of antagonistic activity (10–21 mm) against marine biofilm bacterial strains with least minimal inhibitory concentrations (MIC) and maximum bactericidal concentrations (MBC). The extract showed promising antimicroalgal activity with MIC values ranging between 100 and 400 μg mL^{-1}. The anti-crustacean activity for 50% mortality (LC_{50}) of *Artemia salina* was recorded as 273.77 μg mL^{-1}. An EC_{50} value of 77.03 μg mL^{-1} for the 50% inhibition of byssus production and attachment of mussel *Perna indica* was observed. The crude extract of *S. fradiae* RMS-MSU showed an LC_{50} value of 718.79 μg mL^{-1} for 50% mortality of mussel. A therapeutic ratio (LC_{50}/EC_{50}) of 9.33 indicated the nontoxic nature of the extract. The mollusc foot adherence assay using the limpet Patella vulgata showed 6.66% fouling and 92.96% regaining at 7 mg mL^{-1} after transfer to fresh seawater (Prakash et al. 2015). The soil samples were collected from the Palk Strait [Point Calimere (Kodiyakarai), Vedaranyam, Athirampattinam, Mallipattinam, Manora, and Muthupet mangrove] coastal environments of the Bay of Bengal, Tamil Nadu, India. Totally, 1394 actinobacterial colonies were isolated in all the four culture media used. Among them, 55 morphologically distinguished actinobacteria were documented. The relationship between the soil physicochemical parameters and total actinobacterial population was investigated using correlation-coefficient test with SPSS package. All the actinobacteria were screened for the production of antifouling activity against biofouling bacteria isolated from poultry farms of Perambalur district. Of the 55 isolates, 21 (38.18%) isolates had antifouling activity (Manikandan and Vijayakumar 2016).

11.1.15 Nanotechnological Potential of Mangrove Actinobacteria

Nowadays, nanotechnology is a rapid, upcoming, and multidisciplinary area, where scientists target the manufacture of materials at the nanoscale level (one billionth of a meter or 10^{-9} in size). Nanoparticles have concerned great attention in the recent past due to their interesting physicochemical properties and their applications in

medical, agricultural, and industrial fields (Kim et al. 2007). Synthesis of nanoparticles could be achieved by the physical, chemical, and biological methods. Due to the involvement of toxic chemicals, high investment, power consumption, etc., the physical and chemical methods are not satisfied that enough. Besides, there is a growing need to develop rapid, simple, inexpensive, and environmentally benign nanoparticle synthesis protocols that do not use toxic chemicals in the synthesis. As a result, researchers in this field have shifted to biological systems (plants extracts, microorganisms, and enzymes) for the synthesis of various nanomaterials through reduction methodologies (Song and Kim 2008). Bio-directed synthesis of nanoparticles is used to attain a high range of chemical composition, size, morphology, high monodispersity, water solubility, biocompatibility, and large-scale production.

Certain microorganisms including bacteria, fungi, actinobacteria, and algae can reduce metallic ions to nanoparticles through their biochemical pathways and genetic regulations (Baker and Tatum 1998). It would be fascinating to explore the potential of eukaryotes to synthesize nanoparticles as their genetic makeup and characteristics vary from prokaryotes which would result in nanomaterials with attractive characteristics and applications. In this case, few genera of fungi and actinobacteria have been studied for their synthesis of nanoparticles (Mukherjee et al. 2001, 2002; Balagurunathan et al. 2011; Shanmugasundaram et al. 2013, 2017). Actinobacteria are a group of Gram-positive, filamentous, high G+C content bacteria and are well-known for their ability to produce secondary metabolites than the nanoparticle fabrication (Vimal et al. 2009). Actinobacteria are capable of synthesizing the nanomaterials in either an intracellular or an extracellular manner. Actinobacteria are accommodating in the biosynthesis of nanomaterials with good surface and size individuality showing extensive bio-properties.

Actinobacteria isolated from the mangrove ecosystem of India were exploited mostly for primary and secondary metabolites production. Besides few research groups reported the isolation of actinobacteria from mangrove soils, particularly for the synthesis of metal and metal oxide nanoparticles (Table 11.4).

11.1.16 Energy Research

A mangrove actinobacterium identified as *Streptomyces olivaceus* MSU3 from Manakudy mangrove ecosystem, Tamil Nadu, saccharified various agro-residues such as vegetables, banana, mango peel, sugarcane bagasse, and sugarcane juice followed by bioethanol production. The potential strain exhibited the maximum percentage of saccharification and bioethanol production in sugarcane juice-substituted medium at optimized culture conditions of pH 6, temperature 30 °C, inoculum size 2.5%, and incubation time 72 h, substituted with 2.5% dextrose and 2% urea, respectively, as carbon and nitrogen sources. The ethanol concentration was estimated through HPLC as 76% with a retention time of 2.05 min and % area of 135,694.91 μV/s (Sanjeevkumar et al. 2017).

Table 11.4 Details of nanoparticles synthesized using actinobacteria isolated from Indian mangrove ecosystems

Actinobacteria	Source of isolation	Nanoparticle	Size/shape	Application	References
Streptomyces spp.	Mangrove soil, Pichavaram	ZnO, CuO	100–200 nm	Antimicrobial textiles	Usha et al. (2010)
Streptomyces albogriseolus	Mangrove sediment, Pichavaram	Ag	16.25 ± 1.6 nm, spherical	Antibacterial	Samundeeswari et al. (2012)
Streptomyces sp. BDUKAS10	Mangrove sediment, Pichavaram	Ag	21–48 nm, spherical	Antibacterial	Sivalingam et al. (2012)
Actinomyces sp.	Mangrove soil, Andhra Pradesh	Ag	5–50 nm, spherical	Antibacterial	Narasimha et al. (2013)
Thermoactinomyces sp.	Mangrove soil, Vellapallam, Nagapattinam	Ag	20–30 nm	Antibacterial	Deepa et al. (2013)

11.2 Conclusion and Future Perspectives

India is blessed with unique diversity and distribution of actinobacteria owing to its diverse geographical, climatic, and eco-physiological conditions. Notably, the mangrove counter part of Indian marine realm is the gold mine for novel actinobacteria as documented by various academicians and scientists. The genus *Streptomyces* is predominantly isolated from mangrove ecosystems situated in different states of India. However, they are randomly explored for several diverse bioprospecting potentials, notably for antimicrobial activities, followed by anticancer and enzymatic activity. Research on mangrove actinobacteria in different aspects of environmental cleanup, aquaculture, and nanotechnological potential is meager.

At the global level, there are several novel rare actinobacterial genera reported from mangrove ecosystems from which structurally and functionally new bioactive metabolites have been reported. *Micromonospora* is the largest genus among the rare actinobacteria isolated from mangrove ecosystem which produces a rich source of natural products such as rifamycin, butemycin, and β-carboline. Other genera of rare actinobacteria, namely, *Jishengella*, *Salinispora*, *Saccharopolyspora*, and *Nocardiopsis*, from the mangrove ecosystem have also produced novel secondary metabolites which are potentially useful in the medical and pharmaceutical industry. However, the in-depth literature survey has revealed that mangrove ecosystems in India are still inadequately investigated for actinobacteria. Isolation of rare actinobacterial genera from Indian mangrove ecosystem for bioactive metabolites is a recent happening in the last decade. A few countable number of metagenomic research have further revealed the presence of several rare actinobacteria in Indian mangrove ecosystems remarkably in the Andaman and Nicobar Islands, Sundarban mangroves, and Pichavaram mangroves which are not yet isolated in laboratories through culture-based approaches. Previous studies have also reported the anti-TB (Radhakrishnan et al. 2010, 2011) and anti-HIV (unpublished data) activity of rare actinobacteria isolated from mangroves of Pichavaram and the Andaman and Nicobar islands. There is tremendous scope for investigating the culturable and uncultured (metagenomic) diversity of actinobacteria from Indian mangrove ecosystems due to the availability of selective isolation protocols as well as the advancements in molecular and bioinformatics tools. Based on the excellent track record as mentioned above, there is a great hope for the isolation of novel anti-infective molecules against the life-threatening multidrug-resistant pathogens like methicillin-resistant *S. aureus*, carbapenem-resistant Gram-negative pathogens, and multidrug- and extensively drug-resistant *M. tuberculosis* and viral pathogens. There are still a lot of avenues yet to be explored, from actinobacteria of the Indian mangrove ecosystems with special reference to agricultural, aquaculture, environmental, nanotechnological, and other countless applications. Thus, there are a lot of avenues for judicious and prolific utilization of actinobacteria from the mangrove ecosystems of India; however, it depends on the untiring synergistic efforts by the microbiologists, marine biologists, chemists, biotechnologists, and pharmacologist for interdisciplinary integrated research toward this initiative to bring actinobacteria into the limelight of recognition due to their diverse applications.

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Gut Microbiomes and Their Impact on Human Health

12

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Abstract

The gut microbiome encompasses the large repertoire of microbes in the gastro-intestinal system and their collective symbiotic functions for the host, viz., protection against opportunistic pathogens, body's immune system, extraction of nutrients and energy from diet, fermentation of non-digestible carbohydrates, homeostasis, etc. Further, dysbiosis of the gut microbiome is associated with diverse human diseases including inflammatory bowel disease (IBD), cancer, type 2 diabetes (T2D), obesity, etc. Composition of the gut microbiome has been characterized through a combination of microbial culture techniques and metagenomic approach that helped in understanding the impact of gut microbiome on human health and disease. Moreover, divergences in dietary habits and varied geographical niches have a role in streamlining the diversity among gut microbiomes of different populations. Further, relative increase in *Firmicutes* and decrease in *Bacteroidetes* in the gut of people living in colder climates of higher latitudes are endowed with more storage of energy and fat from a given diet. In the case of neonates, the gut microbiota undergoes transformations and has a major role in nutrition and the development of immune system. Furthermore, the gut microbiota has been used as potential probiotics for improving the intestinal microbial balance.

Keywords

Human gut microbiome · Metagenome · Symbiosis · Dysbiosis · Diet · Geographical niche · Probiotic · Prebiotic · Fecal transplantation

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12.1 Introduction

Gut microbiome is generally regarded as a microbial population in the human gut. Human gut harbors diverse microbiota which forms a complex system that interacts between host and environmental factors (Xu and Knight 2015). The gut microbiota comprises a wide array of bacteria, virus, archaea, and eukaryotes. The gut microbiome as a whole represents about 150 times more genes in their collective metagenome than that of host genome (Qin et al. 2010). To understand the effect of gut microbiota in human health and diseases, identification and characterization of microorganisms are crucial. The combination of culture-dependent and molecular-based sequencing methods has facilitated to understand the composition and population dynamics of gut microbiome (Turnbaugh et al. 2007; Ridlon et al. 2014). In gut, the dominating populations represented by obligate anaerobes, thus, require more nutrients for fastidious growth and are interdependent on each other that render 70–80% of the gut microbiota as non-culturable (Dethlefsen et al. 2007). The gut microbiome community is affected by numerous exogenous factors, of which the long-term diet appears to have the major effect. Although the gut microbiome is resilient, long-term and short-term dietary interventions can alter overall gut community structure. Besides, gut microbial composition is influenced by the host physiology (Backhed et al. 2005; Ley et al. 2005, 2006; Jumpertz et al. 2011). For instance, the obese individual possesses distinct bacterial composition compared with the lean individual that is characterized by a relative abundance of two dominant phyla, i.e., an increase in *Firmicutes* and decrease in *Bacteroidetes*. The gut microbial communities are also mediated by ecogeographical variation. In this regard, population at higher latitudes tends to have larger body mass compared with population in lower latitudes. The *Firmicutes* are relatively more abundant in people living at higher latitudes, whereas *Bacteroidetes* are relatively abundant in lower latitudes (Ley et al. 2005).

The gut microbiota plays many important physiological functions, ranging from regulation of energy metabolism, detoxification, homeostasis, nutrient metabolism, and immunity against pathogens. It also regulates the metabolic mechanisms by controlling the host genes (Lin et al. 2014). The alterations in the gut microbiota composition (dysbiosis) contribute to the development of various chronic diseases including inflammatory bowel disease (IBD), cancer, type 2 diabetes (T2D), obesity, fatty liver disease, lung infection, cardiovascular dyslipidemia, metabolic endotoxemia, etc. Apart from this, the gut microbiota has shown effect on the brain (Holmes et al. 2011). Neonatal intestinal microbiota composition fluctuates and depends on factors such as gestational age, type of feeding, mode of delivery, environmental exposure, and other medical interventions (Penders et al. 2006; Orrhage and Nord 1999; Dominguez-Bello et al. 2010). Furthermore, gut microbiota has been studied for their potential use as a probiotic in relation to a wide range of biological or clinical effects. The most commonly used probiotic microorganisms belong to the genera *Lactobacillus* and *Bifidobacterium* (Lin et al. 2014). The results of various clinical studies have confirmed the synergistic effect of probiotics on gastrointestinal and allergic diseases. probiotics It is evident from several studies that the probiotics are effective in the treatment of diseases such as obesity, insulin

resistance syndrome, type 2 diabetes, nonalcoholic fatty liver disease, and lack of immunity (Markowiak and Slizewska 2017). Prebiotics are non-digestible food ingredients which have beneficial effects on the host by stimulating growth and metabolic activity (Gibson and Roberfroid 1995). Prebiotics have huge potential for modifying the gut bacteria at the level of individual strains or species (Markowiak and Slizewska 2017). In this context, therapeutic applications of gut microbiome dysbiosis have been demonstrated successfully from a donor to a recipient in order to change the recipient gut microbial composition by fecal microbiota transplantation (FMT) (Smits et al. 2013). This chapter describes the overall human gut microbiome, the symbiosis and dysbiosis of the human gut microflora, and their impact on host's health and disease.

12.2 Human Gut Microbiome: Overview

12.2.1 Gut Habitats and Their Inhabitants

The human gut is one of the most recognized densely populated and complex ecosystems. Majority of the population contains mostly obligate anaerobes with less number of facultative anaerobes, archaea, and yeast (Human Microbiome Project 2012). The microbiota in the lumen carry out fermentative process as the gastrointestinal tract possesses anaerobic environment. It has been estimated that the colon of an adult contains approximately $2–5 \times 10^{11}$ bacteria/gram wet weight feces. On an average, the gut microbes utilize 100–200 g/wet weight/day of sloughed intestinal cells, plant polysaccharides, cellulose, starch, and bile components as major substrates for their growth. By using these substrates, the gut microbes produced many short-chain fatty acids (SCFAs) like acetate, propionate. butyrate, etc. which constitute up to 10% of the total caloric input per day (Donohoe et al. 2011). Gut bacteria also produce several metabolites from the fermentation of amino acids (viz., cresol, phenylacetate, and indole) that are toxic to the host (Ridlon et al. 2014). Human gut microbiota consists of more than 1000 phylotypes that are divided into six phyla, including *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* (Human Microbiome Project 2012). Among them, *Firmicutes* and *Bacteroidetes* constitute more than 90% of the total gut microbiota. The major obligate anaerobes in the human gut include *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, and *Ruminococcus*. Similarly, the presence of facultative anaerobic bacteria such as *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, and *Proteus* has been enumerated (Ridlon et al. 2014).

12.2.2 Stomach and Small Intestine (SI)

The gastrointestinal tract microbiota plays a vital role in the synthesis of amino acids required for the host (Raj et al. 2008). The cell density in the intestinal effluent is about 10^4–10^5/mL in the small intestine followed by higher population (10^8/mL) in

the ileum (Walter and Ley 2011). It has been reported that the mucosa-associated bacterial population in the small intestine (SI) represents the genera *Bacteroides* and *Clostridium*, and the lumen contains members of *Enterobacteriaceae* (Wang et al. 2005). Factors which restricted the bacterial biomass in the stomach and SI are low pH of the gastric contents and rapid luminal flow (Kanno et al. 2009). Bile salts and immunoglobulin A (IgA) are highly bactericidal and recognize the dominant microbes present in SI. IgA restrain the microbial penetration into the mucosa which triggers bacterial agglutination in the mucus (Hooper and Macpherson 2010). Following this metabolic process, IgA shapes the normal gut microbiota diversity and prevents the loss of total bacterial population in the proximal small intestine (Suzuki et al. 2004).

12.2.3 Large Intestine (LI)

Properties like less acidic pH, larger volume, low concentrations of bile salts, and slower peristalsis of the large intestine (LI) allow more bacterial proliferation. As a result, the host's immune system permits densities of bacteria beyond 10^{11} cells/g content (Walter and Ley 2011). Like the small intestine (SI), the majority of the mucus-associated microbiota in the large intestine (LI) belongs to the *Firmicutes* and *Bacteroidetes* (Eckburg et al. 2005). Other phyla present include *Actinobacteria*, *Verrucomicrobia*, and a number of less abundant phyla such as the *Proteobacteria* and *Fusobacteria* (Costello et al. 2009; Turnbaugh et al. 2009). Archaea like *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* are also present in the gut (Eckburg et al. 2005). In addition, protozoans and fungi have been reported in the large intestine (Scanlan and Marchesi 2008). Viruses like prophages and phages also play important roles in the gut ecosystem (Reyes et al. 2010).

12.2.4 Archaea of the Gut Microbiome

Methanobrevibacter sp. and *Nitrososphaera* sp. are the major archaea present in the human gut. The two genera are mutually exclusive and coexist in the gut. *Methanobrevibacter* sp. is relatively abundant in the human gut as compared to *Nitrososphaera* sp. (Hoffmann et al. 2013).

12.2.5 Fungi of the Gut Microbiome

Twelve fungal genera were recorded in the human gut. The most prevalent genus was *Saccharomyces*, followed by *Candida* and *Cladosporium*. The phyla *Ascomycota* and *Basidiomycota* are common in the gut (Hoffmann et al. 2013).

12.2.6 Gut Microbial Profile of the Fetus and the Newborn

The infant's gut is colonized by bacteria from the mother and the surrounding environment immediately after birth (Murgas Torrazza and Neu 2011). The contact with the mother's vaginal and intestinal flora during the birth is an important source for the initiation of the infant's gut colonization with the predominant *Lactobacillus*, *Prevotella*, and *Bifidobacterium* (Biasucci et al. 2008; Wild and Krutzfeldt 2010). In the case of cesarean delivery, the direct contact of the newborn's mouth with vaginal and intestinal microbiota does not occur, and as a result, non-maternally derived environmental bacteria play an important role in the infant's gut colonization. So the cesarean baby gets colonized with less diverse microbial flora (Biasucci et al. 2008; Salminen et al. 2004; Wild and Krutzfeldt 2010). Intestinal microbiota plays a characteristic role in the postnatal immune development of the baby (Bjorksten 2004). The cesarean babies are more prone toward many epidemiological diseases like atopic diseases, asthma, type 1 diabetes, and food allergies than the babies born normally (Eggesbo et al. 2003; Laubereau et al. 2004). Thus, the early days' enteric microbiota composition seems to be a very important factor for achieving and maintaining good health in the coming years (Murgas Torrazza and Neu 2011).

12.2.7 Microbial Ecology of the Gut

Scientists revealed that the gastrointestinal tract contains autochthonous and allochthonous members of the microbial community that provides useful framework for understanding the patterns of diversity and stability in the human gut. Autochthonous members are those that occupy the specific niches and form stable population over a long period (Savage 1977). In comparison, the allochthonous members do not possess any specific niches. Relatively stable microbial and viral communities are found to be resistant to blooms of subpopulation, dietary changes, as well as moderate antibiotic doses (Ley et al. 2008; Costello et al. 2009; Reyes et al. 2010; Robinson et al. 2010; Martinez 2014). Evidence suggests that the microbial communities colonizing the large intestine (LI) are dominated by autochthonous microbes to a large degree. For example, *Helicobacter pylori*, detected in the human stomach, are not found in the oral cavity or food and also possess the phenotypic and genomic traits to survive in the stomach (Tomb et al. 1997; Bik et al. 2006). In contrast, taxa representing *Streptococcus mitis*, *Veillonella* sp., and *Granulicatella* sp. found in the stomach and SI also dominate the oral cavity (Aas et al. 2005; Bik et al. 2006; Booijink et al. 2010). Thus, introduction of high numbers of allochthonous microbes might contribute to the chronological variation in the SI. Despite its high temporal variation, the human SI is likely to possess an autochthonous microbiota in addition to *H. pylori* (Walter 2008).

12.3 Study of Human Gut Microbiome

Human gut microbiome consists of a large diversity of microbial communities (Erickson et al. 2012). The determination of the human gut microbiome composition and its role on human health and disease represents a major challenge in the twentieth century (Turnbaugh et al. 2007). With the advancement of high-throughput sequencing techniques, it is possible to analyze the microbiome associated with the diverse environments (Hiergeist et al. 2015). It is believed that 80% of the bacterial species encountered in the human gut are not culturable. This resulted in a lack of interest for culturing microbiome of human gut fastidious anaerobes (Finegold et al. 1974). A purely sequence-based explanation of a specific microbial ecosystem provides valuable information about community content and metabolic potential, but in ecosystem, microbial abundance to function ratio varies to a great degree. For instance, keystone species found in the ecosystem is less abundant but plays a great role in the key metabolic function upon which the entire community structure may rely (Ze et al. 2013). So culture of those less-abundant species is necessary to understand their role in the microenvironment.

12.3.1 Culturable Approach of Gut Microbiome Study

Applications of metagenomics are powerful tools for the investigation of microbial community, but suffer from some key limitations. Therefore, culture-based approaches have been considered for the identification and characterization of microorganisms at the genus or species level. Culture of bacteria is required to understand their role in causing or maintaining healthy and disease states which is not possible through the culture-independent method (Lagier et al. 2012). Limited information on the culturable microbial community has been obtained from the sequence-based approach; thus the culture of microbes is necessary to understand the role of the entire community. Cultivation of microbes in consortia during disease or healthy conditions is also required for better understanding of stochastic interaction in maintaining the host physiology. Several attempts have been made to cultivate the non-culturable bacteria by modifying the growth conditions (media composition, pH, temperature, etc.). In this regard, enrichment media have been used to allow the growth of microbes. For example, the verrucomicrobial organism *Akkermansia muciniphila* was first isolated from feces due to its unusual ability to use mucin as a sole carbon and nitrogen source. In another study, Renesto et al. (2003) had successfully grown the strain *Tropheryma whipplei* by supplementing L-glutamine and other amino acids to the growth medium in order to make the strain culturable based on genomic information.

The second, more shotgun approach to culture yet to be cultured microbes has been developed by Lagier and colleagues and was named “culturomics” (Lagier et al. 2012). In their landmark work, the authors used 212 different culture conditions and demonstrated the culture of 340 different bacterial species including 32 novel species as well as 5 fungal species and a giant virus. Combined with automated

colony-picking technology and high-throughput growth arrays, culturomics represents a powerful technique for cultivation of microbes. Following this, Dubourg et al. (2013) analyzed the microbiota of patients of tuberculosis and the gut microbiota of patients treated with broad-spectrum antibiotics. They reportedly discovered hitherto uncultivated species, together with not yet identified bacterial species. Another study done by Rettedal et al. (2014) used several culture conditions to capture a representative portion of the gut microbiota involved in the maintenance of overall microbial community. In culturable approach, around 88% of the taxonomic group at family level were obtained which is 40% more operational taxonomic units (OTUs) than that found in the non-culturable approach. This demonstrated the advantages of culturable approach to capture the gut microbial diversity (Rettedal et al. 2014). Enhanced knowledge of cultural conditions in association with exploration of techniques like mass spectrometry and pulsed-field gel electrophoresis (PFGE) contribute a lot toward culturomics (Seng et al. 2009). Also microbiologists have developed systems like chemostat or continuous culture to maintain the physiologically relevant conditions (Payne et al. 2012; Venema and Van den Abbeele 2013).

12.3.1.1 Limitations in Culture-Based Approach of Gut Microbiome Study

Traditional culture-based approach still has value but is limited by many factors. Specific nutrient requirements of microbes and time required by some species to grow under sub-optimal conditions are a few of the limitations associated with the culture-dependent approach. The strict anaerobes in the gut live in consortium and follow an interdependent growth.

12.3.2 Culture-Independent Approach

The dominating obligate anaerobes in the gut microbiota and their extreme sensitivity to oxygen, fastidious nutrition, and interdependence on each other result in a small number of gut microbes being culturable (Dethlefsen et al. 2007). Hence, new methods have been exploited for studying non-culturable members of the human gut. Only 10–30% of the gut microbes could possibly be recovered by the culture-based approach, but the selective media used for microbial cultivation cause distortion in the composition of the gut microbiome. The rapid advancement in sequencing technology led to the wider use of sequence-based metagenomics in studying the complex ecosystems such as the gut (Tringe et al. 2005; Tyakht et al. 2013). In non-culturable approach, both 16S rRNA sequence and metagenomics analysis are considered for the study of gut microbiome (Woese and Fox 1977). Further, 16S rRNA sequencing will assist in identifying the representative taxa in the gut, while the whole-genome analysis discovered the functional attributes inside the gut (Lepage et al. 2013; Handelsman 2004).

12.3.2.1 16S rRNA Sequencing

This sequencing technique is a gold standard for which the curated taxonomic database exists to study the phylogenetic relations and identification of new strains in prokaryotes (Vinje et al. 2014). Assessment of bacterial taxa and their relationship with closely related species was introduced by Woese and Fox (1977). The conserved sequence of 16S rRNA genes is divided into nine variable regions (V1–V9), which are important for assessing species richness and in studying the microbial community composition (Giovannoni et al. 1990).

12.3.2.2 Metagenomics

The metagenomic approach deals with the study of collective genomes from an environment; hence it gives an insight into the function of noncultivated microbes present in the ecosystem. Metagenomics was first described by Handelsman in 1998 and employed to analyze the soil and aquatic ecosystems. The first sequence-based characterization was done by obtaining samples from two American healthy volunteers, and a significant enrichment of the gut microbiome associated with metabolism of glycans, amino acids and xenobiotics, methanogenesis, and 2-methyl-D-erythritol 4-phosphate pathway-mediated biosynthesis of vitamins and isoprenoids was demonstrated (Kurokawa et al. 2007). Infant gut is prevalently consisting genes involved in carbohydrate transport and energy uptake from milk, while the adult gut is found to be enriched with genes responsible for energy harvest from diet (carbohydrate metabolism), and bacterial competition (antibacterial peptide transport). This change in gene abundance suggests the human–microbes co-evolution. The less number of genes involved in flagella formation and chemotaxis suggested the microbial commensal depletion due to the development of host immune system. Holler et al. (2014) found the microbiome shifts of predominant commensal bacteria toward *Enterococci* and subsequently recovered from active gastrointestinal graft disease. Qin et al. (2012) analyzed the microbiome of type 2 diabetes patients using metagenomic approach and noted the differences in the gut microbiome composition between healthy and diseased persons.

The European project MetaHit and the American Human Microbiome project contributed to the available reference gene catalog (Qin et al. 2010; Blottiere et al. 2013). In total 3.3 million non-redundant genes in the human gut microbiome have been found by studying 124 European individuals' fecal samples by metagenomics. The gene set was 150 times larger than the human gene complements, which suggested 99% of the human gut community is bacteria (Qin et al. 2010). Lakhdari et al. (2010) investigated intestinal microbial pathways using high-throughput sequencing technology and found that *E. coli* clone could modulate the intestinal mucosal proliferation by activating NFκB. By applying functional metagenomics, it has been found that the secreted and the surface-exposed protein in gram-positive bacteria plays a major role in immune modulation of humans (Dobrijevic et al. 2013).

12.3.2.3 Limitations in Metagenomics

Metagenomic analysis requires a sufficient quantity of DNA which is difficult due to 50–90% host DNA contamination. The functional annotation of metagenomic

sequence entirely depends on the close sequences in the reference database, which is not adequate (Qin et al. 2010). It is also difficult to assign a function only on the basis of sequence similarity (Schnoes et al. 2009). In metagenomics, the study material is the DNA from which expression of functional genes in a given environment is very difficult to determine (Wang et al. 2015). Although metagenomics is a powerful tool for studying non-culturable microbial community, it has limitation in retrieved whole-gene pool. Therefore, metatranscriptomics, proteomics, and metabolomics could be considered as additional platform to exploit the structural and functional activity of the microbes in the gut.

12.3.2.4 Metatranscriptomics

The metatranscriptomics retrieves and sequences the mRNA from microbial community to access types of genes expressed in a particular microenvironment (Gosalbes et al. 2011). Metatranscriptomic studies have been applied to the microbiome isolated from soil and water (Bailly et al. 2007, Frias-Lopez et al. 2008); the information on human gut metatranscriptomic analysis is limited. Mahowald et al. (2009) performed whole-genome transcriptional analysis of colonic RNA prepared from the mice that were germ-free and infected with *Bacteroidetes* and *Firmicutes* and concluded that the gut microbiota illustrates a niche specialization. Similarly, *Bifidiobacterium* found in the gut exhibits differential transcriptional responses in infant depending upon the diet (Klaassens et al. 2009). Study of microbiome from human stool sample evidenced expression of genes responsible for carbohydrate metabolism, energy production, and synthesis of cellular components. In contrast, the reduced level of mRNA was recorded for amino acid and lipid metabolism (Gosalbes et al. 2011). Following this, many untranslated regulatory elements were identified in the gut microbial community from two monozygotic identical twins (Turnbaugh et al. 2010).

12.3.2.5 Limitations in Metatranscriptomics

Limitations in the metatranscriptomics include the lack of possibility in obtaining high-quality RNA; RNAs are very much prone to degradation, and the abundant RNA-like rRNA masks the mRNA of interest, and the database for RNA is also not sufficient. RNA has short half-life making it difficult to analyze the rapid and short-term changes happening in the gut (Simon and Daniel 2011). It is difficult to study the gene expression due to lack of mRNA enrichment in prokaryotes. Moreover, the metatranscriptome database is not so mature; therefore, functional annotation still remained a difficult task in prokaryotes (Wang et al. 2015).

12.3.2.6 Metaproteomics

Metaproteomics deals with the analysis of functional variation of translated proteins in microbiome during colonization, diseased conditions, and dysbiosis (Xiong et al. 2015). The advancement in high-performance mass spectrometry has greatly increased proteome analysis including identification and quantification of proteins (Li et al. 2012). In this regard, the proteome profile of infant feces suggested the infant gut community changes over time with a dominance of the transaldolase

protein synthesized by *Bifidobacterium infantis*. (Klaassens et al. 2007). Further, analysis of the mucosal luminal interface (MLI) collected from 205 mucosal lavage samples including 38 healthy individuals showed extracellular proteins involved in response to stimulus and immune system processes. The proteome profile is significantly related to the biogeography and the individual (Li et al. 2011).

12.3.2.7 Limitations of Proteomics

It is difficult to isolate large amount of proteins due to the charge separation and the loss of samples at each step of isolation, purification, and concentration. Due to insufficient database, the functional annotation is limited as in the case of metatranscriptomics. It is also a difficult task to disentangle the complex proteins synthesized by the gut microbiome (Wang et al. 2015).

12.3.2.8 Metabolomics

Metabolomics is the latest “omics” focused on the study of intermediates and end metabolic products of an organism or a specific biological sample (Klupczynska et al. 2015). This approach provides an opportunity to study the influence of genetic variation, disease, and use of medicines or diet upon endogenous microbiome metabolism. This study involves a comprehensive analysis of small metabolites of size < 1 KDa which are generally carbohydrates, amino acids, lipids, nucleic acids, vitamins, antioxidants, and many other classes of compounds. This field is more related to microbiome phenotype than other omics. Currently, the metabolomics is increasingly used in gut microbiome study. Analytical techniques like nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are being used to analyze the small metabolites. Subsequently, LC-MS and ultra-high-performance liquid chromatography (UHPLC) techniques aid in analyzing the metabolites. In metabolomics, the other approaches include (a) metabolic fingerprinting, (b) metabolic profiling, and (c) targeted metabolomics (Klupczynska et al. 2015). The metabolic fingerprint aims to discriminate between specimens from different biological status like disease-health based on unique pattern characterizing a metabolic state in particular tissue (Blekherman et al. 2011). Metabolic profiling is a non-targeted approach in which common metabolites like amino acids, nucleic acids, lipids, and sugars are quantified. This approach targets to check the changes associated with the known metabolites (Schuhmacher et al. 2013), while the targeted metabolomics focus on a predefined metabolite identification and quantification (Drexler et al. 2011).

The metabolomics is used in diverse fields like toxicology, pharmacology, and widely in medical research to identify the biomarkers of various diseases and biochemical pathways involved in their pathogenesis (Nicholson and Lindon 2008). In this regard, human intestinal disorders like colorectal cancer, irritable bowel syndrome, and inflammatory bowel disease have been studied using metabolomic approach (Phua et al. 2014; Li et al. 2011; Marchesi et al. 2007). Comparative metabolomic study between patients with ulcerative colitis and inflammatory bowel disease with the control healthy individuals showed that the patients had increased quantity of taurine and cadaverine; importantly, the patients showed a

higher bile concentration and lower level of branched-chain fatty acids with no significant changes in short-chain fatty acid and amino acid concentrations (Li et al. 2011). The study of Zhao et al. (2018) demonstrated the enteric changes in the gut microbiome by comparing metabolites by LC-MS and identified 40 metabolites including 9 nucleic acids, 8 organic acids, 4 sugars, 3 alcohols, 3 steroids, 3 lipids, 3 amino acids, and 7 other metabolites.

12.3.2.9 Limitations of Metabolomics

This is the latest among all omics, and it is in its infancy, so it has a few pitfalls. The pitfalls include technical limitations, bioinformatic challenges, and integration with other omics sciences. The metabolomic database is incomplete and insufficient, and a lot of metabolites are not included in the database. Due to insufficient size of database, the metabolomic study remains a challenge that needs to be explored. Separation of microbial metabolites from host metabolites is difficult. The metabolites isolated from the microbiome are mixed, and it is difficult to separate the metabolites of microbes from the host. All approaches possess few limitations in studying the human gut microbiome, so the study between the culture-based and sequence-based gut microbiome could lead to a better understanding of gut ecology.

12.4 Dietary and Geographical Influence on Gut Microbiota

The microbial diversity and composition are highly dynamic and depend upon dietary as well as geographical distribution. With alteration in diet and geographical variations in the gut, microbiota is observed, which indicates the health status of the individual.

12.4.1 Dietary Influence on Gut Microbiota and the Syntrophism Between Them

Studies have revealed that the *Bacteroides* is predominant in individuals consuming high levels of animal protein, amino acids, and fats, while *Prevotella* and *Methanobrevibacter* are high in the individuals eating higher proportion of carbohydrates (Hoffmann et al. 2013). Members of the genus *Nitrososphaera* present in the gut oxidize ammonia and degrade urea, which most probably provides nitrogen to the gut microbial community (Hoffmann et al. 2013). Reports suggest that *Candida* is able to degrade starches, but for that pretreatment with amylase present in the mouth and the small intestine is required (Iannotti et al. 1973). In this manner, *Candida* assists the starch breakdown from the carbohydrate-rich food and liberates simple sugars that finally are fermented by bacteria, such as *Prevotella* and *Ruminococcus*. The by-products produced from the fermentation would then be consumed by *Methanobrevibacter* with the subsequent production of CO_2 and/or CH_4 (Stams and Plugge 2009). The phenomenon of syntrophism is observed in the gut between *Ruminococcus* and methanogens, where the methanogens consume

hydrogen, allowing *Ruminococcus* to produce twice as many ATP molecules from the same amount of substrate (Stams and Plugge 2009).

Environment plays a significant role in establishing and transforming the gut microbiota (Xu and Knight 2015). For example, the physical proximity between humans and companion animals facilitates the acquisition and exchange of microbes. A study of skin, oral, and gut microbiomes showed that the microbial communities of cohabiting family members resemble each other (Song et al. 2013). Various co-evolution studies of mammals and their gut microbiota have found that both gut microbiota composition and functions are adapted to their diet. The overall structure of human gut microbiota is largely driven by long-term dietary effects (Wu et al. 2011). Studies of the fecal microbiomes of human monozygotic and dizygotic twins have been helpful in identifying the effects of diet on gut microbiota controlling for host genetics (Smith et al. 2013). As a whole, diet is among the most easily controlled factors that can potentially constitute the gut microbiota (Delsuc et al. 2014). The ratio of Bacteroidetes and Firmicutes increases in the gut microbiota of people consuming either a fat-restricted or carbohydrate-restricted low-energy diet (Xu and Knight 2015).

12.4.1.1 Bile Acid: Regulator of Gut Microbiome Community Structure

Bile acid is a major regulator of the gut microbiota. In liver cirrhosis, bacterial dysbiosis is linked to low bile acid levels entering the intestine which leads to a significant reduction in gram-positive members of the normal microbiota, such as *Blautia* and *Ruminococcaceae* (Kakiyama et al. 2013; Bajaj et al. 2014). As cirrhosis advances, there is an increase in potentially pathogenic taxa, *Enterobacteriaceae*, which in turn is associated with decreased fecal bile acid levels (Kakiyama et al. 2013). Levels of bile acid entering the large intestine have a profound effect on the major division/phyla-level taxa in the lumen of the gut (Ridlon et al. 2014). Moreover, the mucosal microbial community is significantly indifferent from the community in the lumen, and these differences are correlated with complications of cirrhosis such as hepatic encephalopathy (Bajaj et al. 2014). Plummeting levels of bile acids in the gut favor gram-negative microorganisms, some of which produce potent LPS and include potential pathogens. Increased bile acid levels in the gut appear to favor gram-positive *Firmicutes*, including bacteria that convert primary bile acids to toxic secondary bile acids (Ridlon et al. 2014).

12.4.2 Influence of Geographical Variation on Human Gut Microbial Composition

According to the “Bergmann’s rule,” population in higher latitudes tend to have a larger body mass compared to population in lower latitudes (Bergmann 1847). The *Firmicutes* was found in relatively more abundance in people living in higher latitudes, whereas *Bacteroidetes* was found in relatively high abundance in lower latitudes. Similarly, humans increase their body mass as an adaptation strategy in the colder climates. In the case of obese individuals, the proportion of *Firmicutes*

population increases in the gut with a decrease in the proportion of *Bacteroidetes* (Devaraj et al. 2013). On the other hand, there also exists a positive and negative correlation between *Firmicutes* and *Bacteroidetes* with latitude, respectively (De Filippo et al. 2010). The factors which control the gut microbial composition include the differences in the surrounding environments. These abiotic and biotic factors, amplified by differences in bacterial dispersal and transmission, are likely to affect diversity on multiple levels thereby increasing the differences between microbial communities and the corresponding hosts (Whitaker et al. 2003; Falush et al. 2003). According to various studies, IBD in European populations is associated with an increase in Firmicutes, whereas the Indian populations show either no or only weak signs of differential phylum abundance and diversity among disease conditions (Verma et al. 2010). An interesting fact has emerged from the relationship of *Bacteroidetes* and *Proteobacteria*, which shows age-dependent patterns and a negative correlation across multiple populations, which could be the result of competitive exclusion of *Proteobacteria* by *Bacteroidetes* (Trosvik et al. 2010). The capability of *Bacteroidetes* to digest complex sugars, their ancient symbiotic relationship with their hosts, and their central position in the gut microbiome help to sustain their abundance over time (Backhed et al. 2005; Xu et al. 2007).

12.4.2.1 Population-Specific Biomarkers for Distinguishing Gut Microbiomes

The ability of microorganisms to access host-derived glycans plays a key part in establishing the gut microbial community (Martens et al. 2008; Hooper et al. 1999). The baby microbiome is rich in enzymes involved in the foraging of glycans represented in breast milk and in the intestinal mucosa (mannans, sialylated glycans, galactose, and fucosyl oligosaccharides) as compared to adults (Kurokawa et al. 2007; Koenig et al. 2011). The studies of Yatsunenko et al. (2012) showed that several genes involved in using these host glycans are significantly overrepresented in Amerindian and Malawian babies' microbiomes compared to US baby microbiomes, most notably exo-α-sialidase and α-L-fucosidase (Yatsunenko et al. 2012). These population-specific biomarkers may reflect differences in the glycan content of breast milk. In fact, the representation of these glycoside hydrolases decreases as Amerindian and Malawian babies mature and shift to a diet dominated by maize, cassava, and other plant-derived polysaccharides (Yatsunenko et al. 2012).

Another biomarker that distinguishes microbiomes based on age and geography is urease. Urease gene expression is significantly higher in Amerindian and Malawian baby microbiomes and decreases with age, unlike in the USA, where it remains low throughout lifetime. Urease releases ammonia that can be used for microbial biosynthesis of essential and non-essential amino acids (Metges et al. 1999; Millward et al. 2000).

12.5 Gut Microbiome and Symbiosis

The gut inhabitants maintain a harmonious relationship with the host, in which both the microbial community and the host remains in a positive correlation with each other. As a result of which host homeostasis as well as the host health remain maintained.

12.5.1 Host Homeostasis

Peristalsis, pH, transit time, nutrient availability, host age and health status, and mucin secretion are the important factors affecting the number and diversity of bacterial species found within the gastrointestinal tract (Roberfroid et al. 2010). Under healthy conditions, the beneficial gut microbiota predominates over harmful species, which is termed as "normobiosis." "Normobiosis" plays a central role in gut homeostasis and optimal development of the host. A wide array of important physiological functions of the host's body, including regulation of energy levels and metabolism, neutralization of carcinogens, modulation of intestinal motility, regulation of immunity, protection against pathogens, etc., are controlled by gut bacteria (Sommer and Backhed 2013). Behavior and cognitive functions such as learning, memory, decision-making, and others of the host are also affected by the gut microbiota (Blottiere et al. 2013).

12.5.2 Nutrient Metabolism

Composition of the diet influences the overall community structure of the gut microbiota and the subsequent fermentation products of the non-digested foods affect the host (Lin et al. 2014). Large intestine is involved in the fermentation of non-digested food nutrients such as carbohydrates and some polysaccharides (Ramakrishna and Roediger 1990). The major bacterial genera that play a principal role in this process are *Ruminococcus*, *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Bacteroides*, and others (Roberfroid et al. 2010), which facilitate food degradation and nutrient utilization through fermentation. The intestinal bacterium *Ruminococcus gnavus* possesses a bifunctional α-galactosidase/sucrose kinase enzyme which catalyzes the hydrolysis of melibiose and raffinose into galactose and glucose/sucrose, respectively (Bruel et al. 2011). A functional β-glucuronidase enzyme was found in *Firmicutes* (Gloux et al. 2011). Moreover, the xenobiotic-responsive genes involved in antibiotic resistance, drug metabolism, and stress response pathways were identified in recent studies (Maurice et al. 2013). The catecholamines generated by gut microbes also have a symbiotic effect on gut physiology (Asano et al. 2012).

Diet-derived lipids also affect specific populations of the gut microbiota. Conversely, the bile acid profile of the host can be modulated by the gut bacteria, which in turn influences the host's liver metabolism (Sayin et al. 2013). Proteins and

peptides reaching the colon are fermented by gut bacteria to yield diverse end products, including branched-chain fatty acids, such as isobutyrate and isovalerate, along with ammonia, amines, phenols, indoles, carbon dioxide, hydrogen, sulfur-containing compounds, etc., many of which have been associated with colon cancer and IBD (Parracho et al. 2005; Benassi et al. 2007; Blachier et al. 2010). An increase in the dietary proteins in healthy individuals results in enhanced generation of these toxins (Mafra et al. 2013).

12.5.3 Role of Gut Microbiota in Neonates

In premature babies, the intestinal microbiota plays a crucial role in metabolism, nutrition, immunity, and defense against pathogens. Dysbiosis of the gut microbiota in neonates effects in the occurrence of various diseases in early phase, as well as late in childhood. Intestinal microbiota plays a central role in establishing the early development of the gut's mucosal immune system, both in terms of its physical components and function (Lundin et al. 2008). Bacteria facilitate the production of antibodies from the gut-associated lymphoid tissue, thereby aiding in the process of protection against pathogens (Stappenbeck et al. 2002; Hooijkaas et al. 1984). The immune system does not react with the commensal species present in the gut, and this self-tolerance develops in infancy (Rook 2010). Gut bacteria help in the expression of one of the pattern recognition receptors (PRRs), known as toll-like receptors (TLRs), in various intestinal cell types, including the epithelium (Rakoff-Nahoum et al. 2004). TLRs are able to discriminate between pathogenic and commensal bacteria in invading the gut mucosal layer. These PRRs identify the pathogens that have crossed the gut mucosal barriers and trigger a set of responses that can take action against the pathogens (Ng et al. 2009). The intestinal microflora has developed a pattern of recognition or selective ignorance to self-antigens wherein the gut bacteria do not actually cause the activation of immunological responses, thereby decreasing the likelihood for autoimmunity (Rook 2010).

12.5.4 Intestinal Immune Cell Monitor the Gut Microbiota

Macrophages and dendritic cells (DCs) are located in the intestinal lamina propria where they prevent immunological reactions against commensal bacteria (Franchi et al. 2012; Denning et al. 2007). Gut phagocytes do not produce significant levels of pro-inflammatory cytokines upon stimulation and remain hyporesponsive to commensal gut bacteria (Denning et al. 2007; Kamada et al. 2013). Adaptive immunity is also involved in microbiota homeostasis. For instance, T_h17 cell differentiation gives protection against *Citrobacter rodentium* infection (Ivanov et al. 2009). In addition, *Bacillus fragilis* activates Treg cells which in turn provide protection against *Helicobacter hepaticus* infection (Ito and Simons 2011; Mazmanian et al. 2008). Intestinal damage caused by *Salmonella enteric* and *S. typhimurium* can be attenuated through the enhancement of proliferation of Treg cells by *Bifidobacterium*

infantis (O'Mahony et al. 2008). The epithelial sensing of intestinal bacteria greatly influences the number and diversity of gut microbes through the production of various metabolites (Jin and Flavell 2013; Prescott et al. 2013).

12.5.5 Microbiota Assembly and Breast Milk

Breast milk contains the maternally generated antibodies, which protect the newborns from various infections. In addition to antibodies, breast milk contains cytokines such as IL-10, epidermal growth factor, and antimicrobial enzymes such as lysozyme. Common configurations of microbial communities that occupy the vaginal and cutaneous regions of the mother's body, before and after birth, are helpful to correlate with the development of environmental enteropathy in mothers and their offspring (Kau et al. 2011).

12.5.6 Control of Metabolic Processes by Gut Microbiota Through the Regulation of the Host Genes

Gut microbiota controls a wide variety of host genes. For example, fasting-induced adipose factor (Fiaf) is a circulating lipoprotein lipase (Lpl) inhibitor produced by the intestine, liver, and adipose tissue (Tilg and Kaser 2011). Fiaf is also a mediator of microbial regulation of energy storage (Backhed et al. 2004). It was observed that mice fed with a high-fat diet complemented with *Lactobacillus paracasei* indeed upregulated Fiaf expression in colonic epithelial cell lines (Aronsson et al. 2010). The gut microbiota synthesizes several hydrolase enzymes that can digest complex dietary carbohydrates to monosaccharides and SCFAs such as acetate, propionate, and butyrate (Backhed et al. 2005). Propionate and acetate are ligands for two G-protein-coupled receptors (GPCRs), mainly expressed by intestinal epithelial cells (Brown et al. 2003; Le Poul et al. 2003). One of the two GPCRs is a regulator of host energy balance through effects that are dependent upon the gut microbiota and their metabolic capacity (Samuel et al. 2008).

12.6 Gut Microbiome and Pathogenesis

The human gut microbiome has been directly linked with a wide array of diseases which encompasses obesity, cancer, IBD, etc.

12.6.1 Inflammatory Bowel Disease (IBD)

There is an important role of the gut microbiota in IBDs with contribution to the immunological component of the disease. IBD represents two disorders of chronic intestinal inflammation, ulcerative colitis, and Crohn's disease (CD). Patients with IBD demonstrated a lower diversity of *Firmicutes* and *Bacteroidetes* and an increase

in number of the mucus-invading bacteria *Escherichia coli* (Frank et al. 2007). Genetic studies revealed the role of microbe-host interaction in IBD pathogenesis. The microbe-host interaction is modulated by an underlying cytokine environment, and the disease appears only when the animal is colonized by normal gut bacteria (Kuhn et al. 1993).

12.6.2 Cancer

Gastrointestinal (GI) tumor is the most prominent example of the association of the gut microbiota with cancer. The bacterium *Helicobacter pylori* has a profound relationship with gastric cancer (Suzuki et al. 2009). Colonic cancer is also closely related with gut microbiota. The complex association between the gut genome and the physiological environment of the host and the microbiota results in the occurrence of cancer. Metabolic profiling studies of colon and other cancers showed profound modulation of lipid and sterol pathways and changes in fecal amino acids, short-chain fatty acids (SCFAs), and amines by the gut microbiota (Monleon et al. 2009; Scanlan and Marchesi 2008; Nambiar et al. 2010). Colonic tumorigenesis is a result of shift in microbial ecology which in turn is a consequence of dietary changes and shifts in metabolic expression. The meat-rich Western diets contain sulfur-reducing bacteria (SRB), which increases the risk of colorectal cancer by producing excess sulfide (Christl et al. 1996). The potent promoter of colon cancer is 4-cresol (Ling and Hanninen 1992), whose production is dependent upon the composition of the microbiota, food intake, and pH of the intestinal tract (Smith and Macfarlane 1996). The 4-cresol is synthesized from tyrosine and phenylalanine by the gut microbiota, such as *Clostridium difficile*, and certain *Lactobacillus* strains (Elsden et al. 1976; Yokoyama and Carlson 1981).

Marked changes in the urinary composition of gut microbial metabolites are the causes of several cancers (Kim et al. 2011). Urinary hippurate, 4-hydroxyphenylacetate, and formate are specific markers for ovarian cancer. On contrary, urinary hippurate was found to be one of the strongest discriminators of lung cancer (Slupsky et al. 2010; Carrola et al. 2011). Diet has considerable impacts on both the microbiome composition and the disease. For example, the Western diet alters the colonic proportion of bile acids by intestinal bacteria. Several species of *Clostridium* demonstrate high and low 7α-dehydroxylase enzyme activity, which could represent a novel target for modifying GI cancer risk. In addition, the gut microbiota also contributes indirectly to cancer development via alteration in cholesterol and lipid metabolisms (Holmes et al. 2011).

12.6.3 Type 2 Diabetes (T2D)

T2D is linked with obesity-related insulin resistance. In T2D, the proportions of the Firmicutes, and specifically the Clostridia class were reduced, while the Bacteroidetes and the class Beta-proteobacteria were enriched (Lin et al. 2014).

The ability of the microbiota to influence the expression of diabetes in individuals and the clear impact upon the choline degradation pathway in diabetic subjects emphasize the potential of the microbes to contribute to disease etiology and expression (Holmes et al. 2011).TLRs are a type of pattern recognition receptors (PRRs) that are important in mediating inflammation and immunity. The cell surfaces express enhanced amount of TLRs in patients with obesity, diabetes, and metabolic syndrome (Dethlefsen and Relman 2011; Carvalho et al. 2012). Antibiotic cocktail administration eliminated many of the Firmicutes from gut and resulted in increased insulin activity and glucose tolerance (Devaraj et al. 2013). Lower levels of *Bifidobacterium* sp. contribute to increased gut permeability, which yields increased concentrations of endotoxins in the circulation, thereby, causing inflammation. Loss of TLR leads to changes in gut bacterial profile, which leads to a greater risk of diabetes (Dasu et al. 2010; Jialal et al. 2012; Devaraj et al. 2013).

12.6.4 Obesity

Obesity includes the positive correlation of the gut microbiota with intestinal permeability, systemic quantity of adipose tissue, and body weight. Several studies have shown that decreasing dietary fiber intake altered the overall composition of the gut microflora which finally resulted in obesity and diabetes (Holmes et al. 2011). Consumption of a high-fat diet results in an increase in gram-negative bacteria. Four bacterial mechanisms have been identified to result in excess energy gain by the human body from food – (a) the gut microbiota increases the energy bioavailability due to the conversion of non-digestible food into biochemically absorbable nutrients; (b) the influence of bacterial metabolism to activate triglyceride synthesis; (c) high-fat diets induce the microbial conversion of choline to methylamines leading to a choline-deficient state and induces liver disease; and, (d) the ability of the microbiome in regulating gut gene expression to favor an obese state (Dumas et al. 2006; Backhed et al. 2007). Several experimental results support a pivotal role for the gut microbiome in the pathogenesis of obesity and related disorders (Devaraj et al. 2013). The lipopolysaccharide (LPS) derived from gram-negative bacteria in the gut triggers increased inflammation due to intake of high-fat diet. Mice fed with a high-fat diet showed evidence of pronounced endotoxemia, associated with an increased ratio of gram-negative and gram-positive bacteria. Chronic metabolic endotoxemia induces obesity and insulin resistance (Delzenne et al. 2011; Cani and Delzenne 2010; Cani and Delzenne 2009). A relative abundance of *Firmicutes* and decreased amounts of *Bacteroidetes* were seen in the obese human individuals as compared to the non-obese individuals. After weight loss in human individuals, an increased amount of *Bacteroidetes* (3–15%) and a decreased abundance of *Firmicutes* were observed (Devaraj et al. 2013).

12.7 Probiotics and Prebiotics in Human Gut

Probiotics is the live microbial feed treatment, affecting the host in a positive manner by improving the intestinal microflora, while prebiotics is the use of non-digestible food ingredients that beneficially affect the host by stimulating the growth of intestinal bacteria, thus improving the host health status (Gibson and Roberfroid 1995). Prebiotics can be used as an alternative to probiotics or as an additional support for them. So, both the pre- and probiotics act in a complementary mode with each other and altogether act to improve the host health.

12.7.1 Probiotics

Probiotics are live microbial feed supplements which beneficially affect the host by improving its intestinal microbial balance (Fuller 1989). Various clinical studies confirmed the positive effects of probiotics on gastrointestinal and allergic diseases. Many other clinical studies have shown the effectiveness of probiotic application for treatment of diseases such as obesity, insulin resistance syndrome, type-2 diabetes, etc. Probiotics also demonstrate their positive effect on human health by increasing the body's immunity (Markowiak and Slizewska 2017).

12.7.1.1 Gut Microbiome as Probiotics

Many microorganisms have been used as a potential probiotics. These belong to the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Bacillus*, and *Propionibacterium* (Lin et al. 2014). In addition, the butyrate producer *Roseburia* (Duncan et al. 2006) and the mucin-degrading bacterium *Akkermansia muciniphila* (Everard et al. 2013) have also been reported as potential probiotic organisms. Among them, *Bifidobacterium longum* and *Bifidobacterium breve* have gained interest in the prevention and treatment of acute diarrhea in infants (Di Gioia et al. 2014). Moreover, each probiotic strain has its own explicit properties. The health benefits provided by one particular probiotic strain cannot be mimicked by the other probiotic strains or mixtures of strains (Gerritsen et al. 2011). Nowadays, multispecies probiotic mixtures are becoming popular (Chapman et al. 2011; Timmerman et al. 2004) as compared to single-strain probiotics. Mixtures of probiotic strains have the advantage over single species with higher efficacy (Gerritsen et al. 2011).

12.7.1.2 Mechanism of Action of Probiotics

Probiotics have numerous advantageous functions in humans, which include their capacity of producing vitamins, antioxidants, and defensins against pathogenic competitors (Schachtsiek et al. 2004; Oelschlaeger 2010). Furthermore, probiotics effectively inhibit the development of pathogenic bacteria, such as *Clostridium perfringens* (Schoster et al. 2013), *Campylobacter jejuni* (Saint-Cyr et al. 2017),

various species of *Shigella* (Hussain et al. 2017) and *Yersinia* (De Montijo-Prieto et al. 2015). Several other positive effects of probiotics lie in the digestion processes, treatment of food allergies (Thomas and Greer 2010), candidoses (Kumar et al. 2013), and dental carries (Nase et al. 2001). Probiotic microorganisms are also the natural producers of B group vitamins. They also increase the efficiency of the immunological system and enhance the absorption of vitamins and mineral compounds (Nova et al. 2007; Mishra and Lambert 1996). Some products of probiotics' metabolism may also show antibiotic and anti-carcinogenic properties (Nova et al. 2007).

12.7.1.3 Influence of Probiotics on the Composition of the Gut Microbiome

The probiotic microorganisms are able to manipulate the intestinal microbiota that includes reduction of luminal pH, secretion of bacteriocins, and prevention of bacterial adhesion on the epithelial cells (Fooks and Gibson 2002; Ng et al. 2009). It has been observed that the presence of probiotic microorganism *Lactobacillus plantarum* WCFS1 influences human duodenal mucosal gene expression linked to the establishment of immunotolerance in human adults (van Baarlen et al. 2009). Hence, probiotic intervention has the potential to counterbalance intestinal dysbiosis and thus restore health (Gerritsen et al. 2011).

12.7.1.4 Commensal Gut Microbiota as Therapeutic Agents

The gut commensal microflora can be used as potential therapeutic agents. For example, *Lactobacillus* sp. confers resistance against colitis in IL-10-deficient individuals (Madsen et al. 1999). Probiotic mixtures containing *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* sp. are effective in treating chronic "pouchitis," a complication following surgical intervention for ulcerative colitis (Gionchetti et al. 2000). Moreover, the human commensal *Streptococcus gordonii*, genetically engineered to produce an antibody fragment with antimicrobial properties, can be used to treat the vaginal *Candida albicans* infections (Beninati et al. 2000). Furthermore, microbial signals that can attenuate the activity of the mucosal immune system may be useful in treating IBD (Hooper and Gordon 2001).

12.7.2 Prebiotics

Prebiotics are dietary components that can influence the composition of the human gut such that probiotic microorganisms can predominate (Gibson and Roberfroid 1995). Prebiotics act like dietary fiber-type carbohydrates and enter into the colon in an intact form where they are fermented by the human gut microbiota (Fuller 1989). Prebiotics confer health benefits to the host by stimulating the growth and/or activities of specific bacterial genera in the gut. Prebiotics favor the growth of *Bifidobacteria* and *Lactobacilli* over potentially pathogenic bacteria (Lin et al. 2014). These have the potential for modifying the gut microbiota at the level of individual strains and species (Markowiak and Slizewska 2017). Based on their

chemical structures, prebiotics are classified into two main groups, the inulin-type fructans (ITF) and the galacto-oligosaccharides (GOS) (Martinez 2014). Good sources of carbohydrates such as fruits, vegetables, cereals, and others are the examples of potential prebiotics. Examples of several other prebiotics are tomato, artichoke, banana, *Asparagus*, berry, garlic, onion, green vegetables, and wheat (Parracho et al. 2005; Payne et al. 2012). Lactulose, galactooligosaccharides, fructooligosaccharides, maltooligosaccharides, and xylooligosaccharides are some well-known artificial prebiotics.

12.7.2.1 Mechanism of Action of Prebiotics

The composition and the metabolic activity of the intestinal microbiota are largely affected by prebiotics (Van Loo et al. 2005). Types of microorganisms that are able to use prebiotics as a source of carbon and energy are determined by the molecular structure of prebiotics. Subsequently, end products of prebiotics (mostly carbohydrates) are used by the host as a source of energy (Grajek et al. 2005). The probiotic microorganisms can ferment the non-digested carbohydrates and subsequently produce some compounds, which can inhibit the development of gastrointestinal pathogens (Gibson and Wang 1994).

12.7.2.2 Prebiotics for Humans

The presence of prebiotics in diet confers numerous health benefits. Consumption of vegetables and fruits can considerably reduce the risk of colorectal carcinoma. This effect is attributed mostly to inulin and oligo-fructose. It has been observed that a daily consumption of 12 g of prebiotics reduces the blood LDL level, maintains the correct intestinal pH value, alleviates the symptoms of peptic ulcers and vaginal mycosis, etc. Various studies on humans demonstrated that *Salmonella typhimurium* infections could be neutralized by protective effects of the prebiotic galactooligosaccharide (GOS) (Asahara et al. 2001). Other health beneficial effects of prebiotics include the prevention of carcinogenesis, support of lactose intolerance, etc. Similarly, Buddington et al. (2002) demonstrated that fructooligosaccharides (FOS) can confer protection against *Salmonella typhimurium* and *Listeria monocytogenes* infections. Further, supplementation of 5–15 % inulin and oligofructose had an effect on reduced occurrence of breast cancer in rats. However, these results need to be confirmed in humans (Asahara et al. 2001).

12.8 Conclusions and Future Prospects

A major portion of gut tissue is occupied by extensive volume of bacteria which has so much potential in impacting our health and wellness. There are remarkable evidences suggesting the direct or indirect role of gut microbiota in host immunity, metabolism, and other physiological functions that ultimately affect most of our health (Shreiner et al. 2015). The human gut microbiota throughout their co-evolution provides mechanisms that interact with our bodies in a beneficial way. These interactions may be observed in the form of immunomodulation in

which several mechanisms regulate the immune response in the gut in a healthy or beneficial manner. For example, some microbiota have been used as probiotics which are found to be helpful in aiding digestion and maintaining the integrity of our gastrointestinal tract (Shreiner et al. 2015). The human microbiome project revealed that the gut microbiota composition varies among sex, age, race, or ethnic group (Hollister et al. 2014). Diet and health status of persons are also responsible for gut microbiota composition (David et al. 2014). Microbiota composition also varies according to the location along the gastrointestinal tract and along axial depth (Eckburg et al. 2005; Nava et al. 2011). Despite variation in composition, the gut microbiota shared common core organisms which serve their core function in protein, carbohydrate, and lipid metabolism (Harrell et al. 2012). This core microbiota provides several heath advantages by enhancing the metabolism, immune system, endocrine signaling, and brain function. Bacterial taxa like *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactobacillus*, and others are members of such beneficial bacterial group (Hollister et al. 2014; Mills 2011).

The interest now has shifted toward the potential therapeutic application of gut microbiota. Metagenomic studies have indicated that the microbiota composition, their richness, and diversity in the gut act as health indicators (Claesson et al. 2012; Gill et al. 2006). Studies have also shown that gut microbiomes have the ability to recover from stress; however, continued stress may lead to a decrease in the physiological compatibility (Dethlefsen and Relman 2011; Cho and Blaser 2012). The therapeutic use of fecal microbiota transplantation (FMT) has been getting increasing acceptance due to perception as a natural treatment and its inexpensive implementation (Kelly et al. 2015). But the infectious potential of this therapy arises beyond its perception; long-term studies are in progress to assess the safety of therapeutic use and concomitant decrease in the risk associated factors. Efforts are underway in exploring the role of microbiota composition in various diseases including liver disease, colorectal cancer, esophageal and gastric adenocarcinoma, diabetics, obesity, and autism.

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Importance of Cyanobacterial Taxonomy in Biotechnological Applications

13

Suvendra Nath Bagchi and Prashant Singh

Abstract

Cyanobacteria possess a host of proteases which unlike heterotrophs do not take part in protein nutrition. Instead, they maintain homeostasis of several vital functions, namely photosynthesis, nitrogen fixation, cellular assembly and disintegration, stress acclimation, and defense against predators. Herein, we review the Clp, FtsH, Deg/HtrA, Ctp, and SppA proteases, which under regular and photooxidative stress conditions maintain the integrity of photosynthetic and cytoplasmic membranes, periplasmic proteins, and photosystem particles, including the core complex protein, D1. The HetR protease by coordinating with the Alr3815 protease enables heterocyte differentiation and protection of nitrogenase from oxygen stress. The cell aggregation PteB proteases and caspases regulate the biomass density of cyanobacterial assemblages, and cyanophycinase mobilizes the reserve N, cyanophycin. Macrocyclization proteases mature up the ribosomally synthesized cyclic peptides of cyanobactin class with varied bioactivities. Numerous cyano-proteases listed in the UniProt database are homologues of eubacteria and higher plants with mostly unknown functions but with immense evolutionary significance in understanding the gene flow across bacteria and chloroplasts. Proteases are exclusive and therefore can be tailormade to customize peptide drug synthesis and to formulate food additives and antimalarial, antivirulence, and antithrombotic agents. Notwithstanding these opportunities, taxonomic inadequacy and lack of proper nomenclature have adversely affected different biotechnological application processes. As a remedy, we propose that polyphasic approach of classification and reassessment of old taxonomic status may be necessary before patenting/commercialization of biotechnological processes/products.

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Chaperon · Cyanobacteria · Endemism · Heterocyte differentiation · Photosystem II · Phylogenetic analysis · Polyphasic approach · Protease

13.1 Introduction

Pure cultures of cyanobacteria used for biotechnological applications are maintained in culture collection centers for over decades. The old taxonomic status of these vital organisms has not been thoroughly revised in course of time. To cite an example, *Anabaena* sp. PCC7120 whose complete genome was sequenced for the first time among the filamentous cyanobacteria, upon further assessment was classified as *Nostoc* PCC7120 (Shih et al. 2013). If biotechnological advancements are not accompanied by refinement of taxonomic positions, the consolidated protocols likely to be patented or commercialized can become technically incorrect and challengeable. With over 400 genome sequences already available, proteomics and metabolomics have emerged as new tools for deciphering genes for natural products with biotechnological applications. But, here too, taxonomic misrepresentation deters the interpretations, and such exercises warrant revisions and even criticisms. Understandably, incorrect classification undermines the projects on determining species diversity and subsequent metagenomic analysis of unculturable cyanobacteria. To bridge this gap, the new-generation repositories are attempting to reclassify cyanobacteria in their possession using modern taxonomic approaches and then deciphering upon the spectrum of bioactive metabolites (Ramos et al. 2018).

In this review, we provide elaborate information on cyano-proteases stressing upon their biochemical properties, evolutionary roles, and biotechnological applications. The relevance of such compilation is immense considering the fact that, in "genomic era," proteomic analysis in cyanobacteria would unravel a huge number of uncharacterized proteases with hitherto unknown functions. We also justified as to why a polyphasic approach is appropriate in cyanobacterial reclassification especially where genomic sequences are not available.

13.2 Cyanobacteria and Their Diversity

Cyanobacteria are one of the earliest groups of oxygen-evolving, Gram-negative microorganisms and are documented to have occupied the Earth for at least 3.5 billion years (Seckbach and Oren 2007). They are found in whole range of habitats including aquatic (freshwater, marine, estuaries), terrestrial and aerophytic, and extreme environments like frigid lakes of the Antarctica and hot springs. One of the key features behind their success in time and space is the speedy acclimatization to the inhabiting ecosystems by way of adaptability to changing environmental parameters, viz., nutrients, light, and temperature (Bhaya et al. 2000).

At spatial scales, the distribution pattern of aquatic cyanobacteria (planktic and benthic) is regulated by temperature, concentration of nutrients, water transparency and depth, average solar radiation, conductivity, etc. Soil physicochemical parameters primarily regulate the soil-dwelling cyanobacteria. The geographical distribution patterns of cyanobacteria are also quite varying; while some forms are cosmopolitan, others could be sub-cosmopolitan or endemic (Ribeiro et al. 2018). The cosmopolitan pattern represents a global or multi-geographic distribution. *Cylindrospermopsis* is one of the classic examples that should be considered as a cosmopolitan genus. This is because the originally tropical species of *Cylindrospermopsis* invaded the temperate freshwaters and established themselves due to acclimatization to low temperatures. In marine ecosystems, *Synechococcus* and *Prochlorococcus* are known for their cosmopolitanism. Sub-cosmopolitan distribution is represented by global occurrences but further selection by the environmental conditions for which inhabitants display species-specific adaptations. There are some Pan-tropical bloom-forming species oscillating between the two tropics. Most of the genera falling in this category of distribution pattern are *Microcystis*, *Arthrospira*, *Anabaena*, and *Anabaenopsis*. Among them, the toxic blooms of *Microcystis* and *Anabaena* are more invasive and are more widely distributed. Likewise, the exclusive temperate genera, *Planktothrix*, *Limnothrix*, *Dolichospermum* (*Anabaena*), certain *Anabaenopsis*, and *Cuspidothrix*, are not successful in tropics.

Dispersal of akinete-forming and nitrogen-fixing cyanobacteria contributes to cosmopolitanism. But, endemism (niche-specificity), dispersal hindrances, geographical drifts, and biotic factors restrict free mobility (Ribeiro et al. 2018). Endemism can be pure which means the species evolve and thrive in some exclusive location, or can be relict, which happens due to habitat fragmentation or disturbance and subsequent species extinction except in few locations. *Aphanizomenon manguinii* and *Trichormus subtropicus* are found only in Caribbean islands. *Dolichospermum*, which is known to have worldwide distribution, due to geographical segregation, makes two phylogenetic lineages, one of European and American origin and the other Australian. Apart from the geographical berries, endemism can also be seen in extreme environments, in which adaptability is a key factor behind niche-specific distribution. In Polar Regions aeolian process, salinity and soil chemical characteristics drive the distribution, whereas in thermal springs, water temperature regulates the patterns. In hot springs with geographical distance, thermophilic *Synechococcus* and *Mastigocladus laminosus* populations show genetic divergence. However, in poles, endemism is not unequivocally established. Genetically convergent Antarctic *Leptolyngbya*, *Phormidesmis*, *Phormidium*, and *Oscillatoria* were found in Arctic and other cold biospheres. Here, biotic factors mark significant contribution to endemism. In Antarctica, mosses and foliage suppress the growth of the underneath free-ice terrestrial cyanobacteria. In alkaline hot springs, the non-sulfur bacterium *Chloroflexi* competes with proliferating cyanobacteria. Herbivores also influence cyanobacterial propagation. While copepods facilitate bloom formation the other herbivore, *Daphnia* etc. suppresses the proliferation. Viral infection plays significant contribution in determining success of different strains of marine *Synechococcus*.

The estimated number of cyanobacterial species ranges between 2000 and 8000, comprising over 300 genera. A more realistic picture was derived using a model of discovery curves (Nabout et al. 2013) with a total of 6280 species, of which 43% already described. The unculturable species have not yet been classified with certainty. As a result, while cyanobacteria represent 23.4% of total prokaryotes, merely 15 species are added per year (Dvořák et al. 2017).

13.3 Cyanobacterial Taxonomy and Systematics

Cyanobacteria are morphologically complex with many features which are difficult to distinguish and, thus, are an extremely challenging group to study taxonomically. Some of the reasons for this complex scenario are morphological plasticity, huge amount of ecological flexibility, and incredible genetic diversity. These situations, combined with a complex and varying mode of asexual reproduction, make their classification and taxonomic assessment exceptionally difficult and prone to more frequent errors. An incredible amount of morphological plasticity varying significantly with changes in environmental and culture conditions is one of the biggest factors that have crucially hindered accurate cyanobacterial identification (Komárek and Komarkova 2004) with an estimated 50% of cyanobacterial strains existing in culture collections being identified incorrectly or been assigned to the wrong taxonomic group (Komárek and Anagnostidis 1989). These estimates, though old, may still hold relevance even in the present times as evident from the frequent reclassification attempts and description of new generic entities (Bagchi et al. 2017; Kabirnataj et al. 2018). Alternatively, the level of errors also reflects indirectly the unprecedented amount of heterogeneity and diversity that the cyanobacteria can exhibit.

The cyanobacterial classification has undergone multiple revisions after the first classification system proposed by Bornet and Flahault (1886–1888). The first revised classification scheme was provided by Geitler (1925) and was followed by Frémy (1929), Geitler (1942), and Rippka et al. (1979). The initial classification system of cyanobacteria was thus based on the botanical criterion. Later, it was noted that they possess a prokaryotic cell structure and hence phylogenetically belong to the Bacteria (Stanier and van Niel 1962). The taxonomic classification proposed by Rippka et al. (1979) further formed the basis of *Bergey's Manual of Systematic Bacteriology* which was widely accepted. This resulted in immediate nomenclature problems as the provisions and the rules of International Code of Nomenclature for algae, fungi, and plants (ICN) differ from those of International Code of Nomenclature of Prokaryotes (ICNP). However, attempts have been made in the harmonization of both the codes but only with little progress, and at present, cyanobacterial nomenclature is driven by both ICN and ICNP (Oren and Ventura 2017). Along with the nomenclatural system, the classification of cyanobacteria is also under constant debate.

Further, with the introduction of electron microscopy and molecular characterization, different research groups proposed new schemes of classification in the last 50 years (Drouet 1981; Bourrelly 1970; Otsuka et al. 2001; Casamatta et al. 2005;

Řeháková et al. 2007; Perkerson et al. 2011). A lot of revisionary works that were undertaken in recent years have been crucial in shaping up our current knowledge of cyanobacterial diversity and taxonomy (Anagnostidis and Komárek 1985, 1988, 1990, Komárek and Anagnostidis 1986, 1989, Büdel and Kauff 2012). On having a look at some of the recent works on cyanobacterial diversity and phylogeny from different and varied ecosystems, it has been observed that a lot of studies have been conducted worldwide using different techniques and methodologies (Giovannoni et al. 1988; Urbach et al. 1992; Wilmotte, 1994; Versalovic et al. 1994; Neilan et al. 1995; Lee et al., 1996; Nelissen et al. 1996; Lyra et al. 1997; Turner 1997; Zehr et al. 1997; Rasmussen and Svenning 1998; Lehtimäki et al. 2000; Castenholz 2001; Rudi et al. 2000; Garcia-Pichel et al. 2001; Suda et al. 2002; Neilan et al. 2002; Henson et al. 2004; Lyra et al. 2005; Rajaniemi et al. 2005; Sihvonen et al. 2007; Berrendero et al. 2008; Komárek 2010, 2013, 2016; Thomazeau et al. 2010; Suradkar et al. 2017; Bagchi et al. 2017; Kabirnataj et al. 2018). In terms of taxonomy, it has been evidently noticed that, since 2000, more than 60 genera of cyanobacteria have been described. Thus, on the international platform, the work on cyanobacterial taxonomy in the past decades can be termed revolutionary.

For a long time, the identification and taxonomy of cyanobacteria was based usually on the visible morphological and some ecological parameters, but with the advent of modern sequencing and phylogenetic approaches, it has been clearly recognized that the phenotypic characters alone cannot be a suitable tool in the modern cyanobacterial taxonomy (Castenholz 1992; Komárek 2005). The current classification scheme which is widely accepted by cyanobacterial taxonomists across the globe has been proposed by Komárek et al. (2014) using the polyphasic approach followed by multilocus molecular characterization of the conserved sequences, namely, *nif*, *rpoC1*, *gyrB*, *hetR*, *rpoB*, *rbcLX*, *cpcBA-IGS*, outer membrane efflux protein (OMEP) region, and the 16S-23S internal transcribed spacer (ITS) (Rott et al. 2018; Mareš 2018; Moten et al. 2018). The primary aim of the authors was to establish a classification system that would reflect the evolutionary history and include monophyletic taxa. The taxonomic positioning of some of the traditional species or genera was revised in this scheme as the phylogenetic positioning did not correspond to the morphological descriptions. Since this classification closely reflects the phylogeny, it can be considered to be superior to the older systems. The combination of these complicated but modern methods along with the thorough evaluation of traditionally well-defined morphological and ecological parameters, followed by the subsequent reevaluation and redefinition of modern typical characters and markers of cyanobacterial entities in various biotopes and environmental deviants, is thus an unavoidable taxonomic marker for correct primary classification. The polyphasic approach has also largely resolved confusions on systematic position at the level of morphological, ecological, and genetically cryptic species and other subgeneric entities (Dvořák et al. 2017; Komárek 2018). Consequently, the classical coccoid (*Chroococcales*) and filamentous (*Oscillatoriales*) lineages were regrouped into a new order, *Synechococcales* (Dvořák et al. 2017). Another highlight of this system was the unification of heterocystous cyanobacteria into a single order, *Nostocales*. However, recent studies have indicated the order

Nostocales to be phylogenetically complex (Berrendero Gómez et al. 2016; Bagchi et al. 2017; Kabirnataj et al. 2018; McGregor and Sendall 2017a, b; Shalygin et al. 2017; Dvořák et al. 2017).

The phylogenetic complexity within the order *Nostocales* may be attributed to the high degree of polyphyly observed not only at the genera level but also at the family level. Many old and well-established genera like *Nostoc*, *Scytonema*, *Calothrix*, *Rivularia*, *Anabaena*, *Westiellopsis*, and others have been shown to be polyphyletic (Berrendero Gómez et al. 2016; Zapomělová et al. 2016; Bagchi et al. 2017; Kabirnataj et al. 2018; McGregor and Sendall 2017a, b; Shalygin et al. 2017). In order to achieve monophyly which is the fundamental goal of the cyanobacterial taxonomists, these polyphyletic genera are being continuously revised using the polyphasic approach. This has resulted in both the description of new genera (Berrendero Gómez et al. 2016; Bagchi et al. 2017; Kabirnataj et al. 2018; McGregor and Sendall 2017a, b; Shalygin et al. 2017) and also the unification of existing genera (Aguilera et al. 2018). Moreover, intermixing between the traditional families has been observed (Vaccarino and Johansen 2011; Komárek 2013, Shalygin et al. 2017), and this has further aggravated the phylogenetic complexity within the heterocystous clade. In a recent study, *Kyrtuthrix* has been reclassified from the family *Scytonemataceae* to *Rivulariaceae* on the basis of phylogenetic relatedness (León-Tejera et al. 2016). Constantly changing classification system and lack of consensus phylogenetic scheme have plagued the cyanobacterial taxonomy. In order to address this issue, it is essential to conduct additional taxonomic and phylogenetic studies on the filamentous heterocystous cyanobacteria at the lower as well as higher taxonomic levels.

13.3.1 *Nostoc* as a Taxonomic Enigma: Problems, Past Trends, and Current Shape of *Nostoc* Taxonomy

The first description of *Nostoc* dates back to the late nineteenth century (species type: *Nostoc commune*) (Bornet and Flahault 1888), and since then, more than 300 species of *Nostoc* have been documented from almost all the geographical realms of the world, thus making *Nostoc* almost omnipresent. The genus *Nostoc* is an incredibly complicated case among the filamentous unbranched heterocystous cyanobacteria. The absence of discrete morphological diacritical features has made it one of the most challenging case studies using morphological data only. Widespread occurrence and presence of a huge number of morphotypes and ecotypes is again one of the contributing factors to the complexities of *Nostoc* taxonomy. Due to the abovementioned reasons, along with few other contributing factors, the taxonomy of *Nostoc* has seen multiple revisions and creation of some new generic entities in the recent few years (Řeháková et al. 2007; Hrouzek et al. 2013; Genuário et al. 2015; Bagchi et al. 2017). In phylogenetic perspectives, it is well known and proved that *Nostoc* is a polyphyletic genus (Rajaniemi et al. 2005; Hrouzek et al. 2005; Papaefthimiou et al. 2008; Fernandez-Martinez et al. 2013). In simpler terms, this polyphyletic origin of *Nostoc* indicates the presence of many new clades outside the *Nostoc* sensu stricto clade which in turn makes the entire *Nostoc* assemblage very

diverse and heterogeneous. With an overall aim to achieve this much desired monophyly within the genus *Nostoc*, many studies have hence strongly suggested the revision of this complex genus which has ultimately led to the establishment of new monophyletic genera like *Mojavia* (Řeháková et al. 2007), *Desmonostoc* (Hrouzek et al. 2013), *Halotia* (Genuário et al. 2015), and *Aliinostoc* (Bagchi et al. 2017) that are morphologically very much similar to *Nostoc* but have clustered distantly with strong consistency from the *Nostoc* sensu stricto clade. Thus, it is envisaged that the best suitable way to solve the taxonomic problems of the genus *Nostoc* is to adopt the polyphasic approach with the molecular and phylogenetic assessment being the primary level and the morphology, ecology, and physiology being the secondary level parameters. It is also recommended for the entire heterocystous cyanobacteria that all the abovementioned approaches in sync with the polyphasic approach must be adopted judiciously as per the demands of the case (Řeháková et al. 2007; Hrouzek et al. 2013; Genuário et al. 2015; Bagchi et al. 2017; Komárek 2013; Komárek et al. 2014; Bohunická et al. 2015; Berrendero et al. 2016; Shalygin et al. 2017; Hauer et al. 2014; Suradkar et al. 2017).

13.3.2 *Anabaena* as another Taxonomically Complex Genus

The genus *Anabaena* (Bory ex Bornet et Flahault 1888) is a complicated case of cyanobacterial taxonomy which has undergone considerable revisions in the past few years leading to the creation of new genetic entities (Komárek and Zapomělová 2007, 2008; Wacklin et al. 2009; Zapomělová et al. 2009, 2012). In historical context, the genus *Anabaena* broadly consisted of a large spectrum of planktic species (Geitler 1932, Elenkin 1938, Starmach 1966). With the advent of better sequencing methods, enhanced understanding of the polyphasic approach, and more streamlined phylogenetic evaluations, it was found that the benthic mat-forming forms and the gas vesicle-forming planktic types were different. As a result, the planktic species of *Anabaena* were separated into the genera *Dolichospermum* and *Sphaerospermopsis* with the phylogeny interestingly indicating them to be closer to the genus *Aphanizomenon*. On the other hand, it was also established that the non-gas vesicle-forming strains clustered more closer to the genera *Trichormus*, *Nostoc*, *Cylindrospermum*, and *Wollea*, though doubts over the exact placement still exist. Furthermore, the strains with narrowed ends were also established as a new genus *Chrysosporum*. Another interesting offshoot of the *Anabaena* clade is *Macrospermum* (Komárek 2008) which is characterized by the possession of a subsymmetrical filament structure and a special type of large akinete, forming metaphytic mats in tropical regions. The establishment of the new genera *Dolichospermum*, *Sphaerospermopsis*, and *Chrysosporum* (Zapomělová et al. 2009, 2011, 2012, 2016) was also confirmed phylogenetically once more by Kust et al. (2015). Notwithstanding though, it may be very difficult phylogenetically to distinguish *Dolichospermum* from *Aphanizomenon*, and more resolved molecular techniques such as genome-wide comparison may be required to further resolve the overlap and divergence between the two genera (Li et al. 2016). Driscoll et al.

(2018), based on complete genome comparison, changed the notion of divergence of new genera from *Anabaena* and emphasized to the facts that *Anabaena* and *Aphanizomenon* are highly intermixed, *Dolichospermum* should not be considered a new genus, and the genera *Chrysosporum* and *Sphaerospermopsis* diverging from *Anabaena* need further revision. Not only the planktic genera, in a study that focused on benthic strains of *Anabaena*, it was emphasized that one of the major problems for the confusions in the taxonomy of non-planktic *Anabaena* was the lack of adequate morphological data of the strains whose 16S rRNA gene sequences were present or phylogenetic evaluation was satisfactory (Komárek 2005, 2008; Skácelová and Zapomělová 2010; Mareš 2010; Halinen et al. 2008; Kust et al. 2015). At the present moment, it is essential and in fact recommended to conduct broader surveys of both planktic and non-planktic forms of *Anabaena* especially in context of the characterization of morphotypes and ecotypes as this alone seems to be the only way to fully understand and rectify the problems of the genus *Anabaena* and its related taxa.

13.3.3 Importance of Studying the Genera *Nostoc* and *Anabaena* in Taxonomic Perspectives and Biotechnological Ventures

Dedicated taxonomic assignments on *Nostoc* and *Anabaena* are the only way through which the economic usage and biotechnological exploration of these two genera can be envisaged. These planned studies, though basic in nature, hold the key for a lot of applied potential as these kinds of work will serve as reference studies and pilot studies for many of the future works that will deal with economic and applied usage of cyanobacteria. Understanding the basics of the genera *Nostoc* and *Anabaena*, with both being well-known nitrogen fixers and photosynthetic models, is actually the only way out for sustained usage in agricultural biotechnology, where biofertilizer technology has been harping crucially on the proper identification of pertinent cyanobacteria. In the field of biotechnology, the well-characterized strains of both these genera could be used for developing enzymes, antibiotics, antivirals, cosmetics, colorants, and even nutraceuticals. Proteases are important for primary and secondary metabolism and have structural and evolutionary importance, and hence, their biotechnological applications are imminent. It must be understood that biotechnology and applied usage harps crucially on the strain that is being exploited, and hence, proper identification and validation of the strain is indeed an essentiality which in any circumstance cannot be ignored.

13.4 Importance of Proteases in Cyanobacteria

According to the endosymbiosis theory, cyanobacteria are considered as the progenitors of plant chloroplasts where the complexity is mirrored in the diversity of proteases existing in cyanobacteria. A majority of the proteases are homologues of bacterial or the chloroplastic proteases. Proteases in proteobacteria occurred mostly

as homo-oligomers. In cyanobacteria, paralogous components developed and hetero-oligomeric structures evolved. In higher plants, more heterogenic oligomers with supportive proteins and several inactive components increased the complexity. Another feature was the organelle-specific subcellular localization and positioning in specific suborganellar locations (Nishimura et al. 2016). The following list compiles the types of proteases in cyanobacteria along with their structure, function, and homology with bacterial and/or plant proteases:

13.4.1 Clp Proteases

The Clp proteases (*c*aseino*l*ytic *p*rotease) are highly conserved ATP-dependent serine proteases present in bacteria and eukaryota (Yu and Houry 2007). In cyanobacteria, the Clp holoenzyme consists of ten distinct Clp proteins: four heat shock proteins (HSPs)/chaperons (ClpB1-ClpB2, ClpC, and ClpX), three ClpP proteins (ClpP1–ClpP3), a ClpP-like protein termed ClpR which lacks the catalytic center, and two adapter proteins (ClpS1–ClpS2) (Andersson et al. 2009). In unicellular non-nitrogen-fixing cyanobacterium, *Synechococcus elongatus* PCC7942 (henceforth *S. elongatus*) Clp proteases are found in two distinct heteromultimeric configurations. In the first configuration, ClpP1 and ClpP2 made the core tetradecameric proteolytic complex in the form of two heptameric rings comprising alternating ClpP1 and ClpP2 in the arrangements 4ClpP1-3ClpP2 and 3ClpP1-4ClpP2. Another configuration of the double ring of 4ClpP1-3ClpP2 is also possible. The ClpP1/ClpP2 complex associates with a hexameric chaperon protein, ClpX. The ClpX later binds with the hexameric ClpS1 or ClpS2 adapter proteins. In the second configuration, ClpP3 and ClpR alternate and build a core tetradecameric proteolytic complex comprising two identical heptameric rings of 3ClpP3-4ClpR. The heteromeric ClpP3/ClpR complex unites with another hexameric chaperon ClpC/HSP100 and subsequently with ClpS1 or ClpS2 adapter proteins (Mikhailov et al. 2015).

The Clps were shown to intricately involve in overall growth and stress tolerance, especially under high light, cold, and UV-B irradiation. In *S. elongatus*, Clp proteases play a role in steady-state growth and long-term acclimation to various stress conditions (Stanne et al. 2007). In an interesting finding, deletion of *clpP1*, *clpP2*, or *clpX* genes in *S. elongatus* resulted in enhancement of circadian period. On the other hand, their overexpression brought down the cycle time. The Clp proteases were also involved in regulating cell division and septum formation. The regulation was shown to be mediated through an expression of "r" (ribosomal)-protein subunit genes. Posttranslational downregulation of the r-subunit proteins due to the chaperon activity of ClpX brought down the transcription of an array of proteins that are involved in circadian rhythm and cell division (Imai et al. 2013).

The role of Clp is particularly significant in another unicellular cyanobacterium, *Synechocystis* sp. PCC6803 (henceforth *Synechocystis*), which acclimatizes to nitrogen-starved conditions by degrading the large light-harvesting complex pigment proteins, the phycobilisomes. It has been demonstrated by Baier et al. (2014)

that under N step-down two phycobiliprotein degrading genes *nblA1* and *nblA2* are expressed. The encoded linker protein Nbl1 (*n*on-*bl*eaching protein *A*) that makes a ternary complex with the chaperon ClpC/HSP100 phycobiliproteins facilitates degradation process.

In higher plants, Clp proteases represent major housekeeping proteolytic enzymes in the chloroplast stroma for maintaining cell homeostasis and removing polypeptides that are denatured either spontaneously or as a result of external stress conditions (Halperin et al. 2001). It has been shown that in *Arabidopsis thaliana* the Clp protease core consists of five ClpP and four ClpR paralogues. Considering the endosymbiotic origin of chloroplast, the cyanobacterial ClpP3/ClpR proteolytic core seems to be the ancestor of plant ClpP/ClpR core (Stanne et al. 2007).

The malaria parasite *Plasmodium falciparum* possesses a protease (PfClpP) and a chaperon/HSP (PfClpC) which are homologues of cyanobacterial ClpP and ClpC. The plasmodial ClpP/ClpC protease complex is found to be localized in apicoplast and is responsible for the development of functional apicoplast. This is a vital process that is required at late trophozoite and early schizont stages of asexual blood life cycle and survival of the parasite inside the host (Rathore et al. 2010). The inhibitors of animal serine proteases and β-lactone compound "U1" inhibit activity of PfClpP, thereby affecting apicoplast growth and segregation. Due to similarity with plasmodial ClpPs, the cyanobacterial ClpP can be used as model enzymes for proteolytic perturbation, and the lead compounds can further be exploited for the development of antimalarial drugs (Ng et al. 2017).

13.4.2 Deg/HtrA Proteases

Under high light intensity, the D1 (PsbA) protein from the PSII reaction center gets degraded. In *Synechocystis*, this photo-damaged D1 protein is cleaved primarily by a serine-type ATP-independent protease designated as Deg (*deg*radation of periplasmic proteins) protease (Funk et al. 2001). The Deg family belongs to ATP-independent serine endoprotease which is widespread among prokaryotes and eukaryotes.

The Deg/HtrA (*h*igh *t*emperature *r*equirement *A*) family includes three homologues in prokaryotes such as *E. coli* and *Synechocystis*: HtrA (DegP), HhoA (*H*tr *h*omologue *A*; DegQ), and HhoB (DegS). In other cyanobacteria, two to five Deg/HtrA proteases are known to be present. The Deg proteases within themselves show remarkable sequence homology (47–58%). The three *Synechocystis* Deg proteases were either localized in periplasm or integrated through transmembrane proteins with outer membrane. The Deg proteases due to hydrophobic bonds have a tendency to trimerize, and moreover, due to the presence of extensive PDZ (*p*ost-synaptic density of 95 kDa *d*iscs large and *z*onula occludens 1) domains, the trimers can take higher oligomerization states. HhoA protein trimerize state, for example, again dimerizes to form a hexameric cage (Hall et al. 2017). Primarily, the Deg proteins play a role during photooxidative D1 protein degradation. The HhoA/HhoB proteases have also been implicated in the degradation of phycobilisome and major light-harvesting antenna proteins (Zienkiewicz et al. 2012). Another important role

of Deg proteases is like chaperons inside the periplasm, protecting cells from protein aggregation and facilitating protein secretion. At a later stage of growth, cyanobacteria start to secrete out the periplasmic Deg proteases (Lam et al. 2015).

The cyanobacterial Deg proteases display a high degree of homology with three luminal and one mitochondrion-located *A. thaliana* Deg proteases (AtDeg1, AtDeg5, AtDeg8, and AtDeg14) (Cheregi et al. 2016).

Inactivation of Deg proteases is also accompanied by loss of virulence in some of the bacterial strains. Incidentally, these are the periplasmic Deg proteases that have a tendency to exude. Secretion of Deg proteases is one of the major mechanisms of virulence in these bacteria, and concomitantly, inactivation of such proteases is part of the abatement of virulence, and hence, agents that inhibit the bacterial Deg protease eventually can prevent pathogenesis. In this context, cyanobacterial Deg protease which is also a secretary protease similar to the pathogenic bacteria can be an excellent model enzyme system to develop antibacterial drugs (Cheregi et al. 2016).

13.4.3 FtsH Protease

FtsH (*f*ilamentation *t*emperature-*s*ensitive *H*) is a membrane-bound ATP-dependent metalloprotease. In *Synechocystis*, four FtsH homologues, notably FtsH1-4, have been isolated and identified. The most common FtsH configuration is the hexameric complex comprising alternating subunits of FtsH2 and FtsH3, which is embedded in the thylakoid membrane. The other FtsH complexes comprise the hetero-oligomeric FtsH1/FtsH3 located in the cytoplasmic membrane. This amount of this complex is much less in comparison with the FtsH2/FtsH3, but nonetheless, it has a much more vital role to play in cell viability. Yet another FtsH arrangement found in the cells is the homo-oligomeric FtsH4 complex anchored with both cytoplasmic and a thylakoid membrane and is considered indispensable for viability (Shao et al. 2018).

The PSII complex found in oxygenic photosynthetic organisms is susceptible to damage by UV-B irradiation and undergoes repair in vivo to maintain its activity. The FtsH proteases have been reported to be involved in the degradation of the damaged D1 protein of PSII complex so that new proteins can replace (Boehm et al. 2012). Apart from D1 FtsH also degrades the unassembled membrane proteins and some soluble proteins (Shao et al. 2018). In fact, in *Synechocystis*, the FtsH1/FtsH3 complex has a major role to play for survival under iron limitation. The protease complex degrades the Fur (*f*erric *u*ptake *r*egulator) repressor and eventually induces transcription of IsiA (*I*ron *s*tress-*i*nduced protein *A*) protein which is an integral component of the PSI supercomplex under iron-deficient conditions. Further, it is believed that not FtsH1/FtsH3, but FtsH2/FtsH3, are responsible for the repair of the D1 protein and maintenance of PSII integrity (Krynická et al. 2014).

Apart from maintaining the integrity of photosynthetic apparatus under stress conditions, FtsH proteases also indirectly facilitate the photosynthetic carbon assimilation. Under HCO_3^- limitation, the cellular level of CO_2 concentration mechanism (CCM) complex in *Synechocystis* is reported to be maintained by FtsH protease

(Krynická et al. 2014). This protease increases affinity of Rubisco for carbon dioxide (CO_2) through a CO_2 concentrating mechanism. CCM eventually increases both CO_2 and HCO_3^- uptake, thus elevating CO_2 concentration in carboxysomes (Badger et al. 2006).

Several of the PSII repairing FtsH proteases in cyanobacteria and chloroplasts are orthologues of each other. For example, chloroplastic FTSH2 and FTSH5 of *A. thaliana* are the orthologues of FtsH2 and FtsH3 of *Synechocystis*. In *Arabidopsis*, a THF1 (*th*ylakoid *f*ormation *1*) protein was required for the assembly of FTSH2 and FTSH5 complexes with PSII supercomplex. In almost a similar fashion in *Synechocystis* too, a cyanobacterial homologue of the THF1 peptide, called Psb29, was required for anchoring of FtsH2 and FtsH3 proteases with PSII particles. Cyanobacterial FtsH proteins therefore can be treated as a model to understand the molecular basis of PSII biogenesis in chloroplast (Bečková et al. 2017).

Masuda et al. (2018) have described about an interesting role of FtsH proteases in diel changes in abundance of PSII and PSI in marine unicellular nitrogen-fixing cyanobacterium, *Crocosphaera watsonii* WH8501. In order to ensure that oxygen-sensitive nitrogenase remains functional, the oxygen evolution cycle is temporarily separated. As an adaptive mechanism, nitrogen fixation operates in dark when there is a massive drop of PSII activity. Relative to light period, in darkness, FtsH overaccumulates. This transient change in protease ensures degradation of PSII proteins, especially the D1 and D2, so that N_2 fixation remains unaffected by oxygen.

13.4.4 HetR Protease

Upon nitrogen deprivation, some of the vegetative cells get differentiated to heterocytes for nitrogen fixation. This process of differentiation is complex and involves many genes encoding the transcriptional factors such as HetR (*het*erocyte differentiation *R*) and NtcA nitrogen-responsive regulatory protein). HetR is an autoregulatory serine-type protease with DNA-binding activity present in heterocystous and some nonheterocystous cyanobacteria. The HetR protease is reported to degrade a repressor protein and alleviate transcription of heterocyte specific genes for nitrogen fixation (Dong et al. 2000). HetR seems to be a specific protease cleaving the calcium-binding protein CcbP in heterocyte-forming cyanobacteria, thereby releasing Ca^{2+}, which is necessary for heterocyte development (Shi et al. 2006). NtcA is considered a global nitrogen regulator present in both unicellular and filamentous cyanobacteria (Frias et al. 1994). Shifting from a nitrogen-replete condition to a nitrogen-depleted condition in *Anabaena* sp. strain PCC7120 (henceforth *Anabaena* 7120) resulted in upregulation of *hetR* gene transcription, and the transcripts were present mostly in those cells that would become mature heterocytes and in pro-heterocytes (Dong et al. 2000). At this point, NtcA and 2-oxoglutarate, which is a signal of high C-to-N ratio, upregulate another gene, *nrrA*, and the product NrrA (*n*itrogen-responsive *r*esponse *r*egulator *A*) positively controls the *hetR* gene expression.

During nitrogen step-down, HetR oligomerization has a significant role to play in its activity during heterocyte development. A tetrameric HetR was the most active form of the enzyme, which peaked with maximum *hetR* expression. Consequently, extensive phosphorylation also takes place, and under this state, the HetR protein exhibited the maximum DNA-binding ability. Later studies revealed that, it is the global transcriptional activator's role of HetR rather than its proteolytic activity that is responsible for activation of a cascade of genes, which leads to the heterocyte differentiation (Valladares et al. 2016). Flores et al. (2018) presented an account of *modus operandi* of HetR by way of upregulation of the expression of *patA*, *hetC*, and *patS* genes for pattern formation; *hepA* gene for Hep (*h*eterocyte *e*nvelope *p*olysaccharide) formation and *hglT* and *devBCA* genes for Hgl (*h*eterocyte-specific *gl*ycolipid) formation. PatS (*pat*tern formation *S*) is supposed to diffuse in adjoining vegetative cells where it serves as a negative regulator of the *hetR* gene, ensuring that HetR is not built up in vegetative cells, thus enabling spacing between the heterocytes.

13.4.5 Alr3815 Protease

Alr3815 is a heterocyte-centered protease in *Anabaena* 7120. The protease consists of three domains: a calcium-requiring cysteine proteinase domain and two bacterial pre-peptidase C-terminal domains (Yang et al. 2011). The Ca^{2+} level in heterocytes remains higher than the vegetative cells. The Alr3815 protease was activated by internal calcium buildup during heterocyte differentiation, and the protease caused effective degradation of the D1 protein, an activity which eventually perturbed the PSII activity inside heterocytes. As a result, oxygen could not be liberated in heterocytes. This important event becomes necessary to create anaerobic conditions required by nitrogenase to perform nitrogen fixation as it is an oxygen-sensitive enzyme. A mutant of the *alr3815* gene, designated Strain-322 was deficient in the Alr3815 protease and was also impaired in N_2 fixation because the PSII was operational causing high pO_2 inside heterocytes.

13.4.6 Ctp Carboxypeptidase

The Ctp family of carboxypeptidases is grouped under the category of *C*-terminal processing peptidases. The Tsp (*T*ail-*sp*ecific) peptidase from *E. coli* belongs to this class (Silber et al. 1992) which is involved in the degradation of mistranslated proteins. The family of *C*-terminal processing proteases of *Synechocystis* consisted of CtpA, CtpB, and CtpC that are homologous to bacterial Tsp peptidase. The CtpA was essential for the assembly of the manganese cluster of PSII complex (Karnauchov et al. 1997). Cyanobacterial *ctp* genes shared more than 30% identity with those of *E. coli* and encoded soluble proteins that resided in the periplasm (Zak et al. 2001). A comparison between wild type and a *ctp*A-deficient mutant of

Synechocystis unable to grow under photoautotrophic conditions due to impairment of *C*-terminal processing of D1 protein revealed that, this peptidase is involved in biogenesis of the photosynthetic machinery (Shestakov et al. 1994). It became clear that CtpA was responsible for processing of D1 protein in *Synechocystis*.

D1 protein is synthesized as a precursor, pD1, which is integrated into thylakoid membranes, and several proteins including the D2 protein get associated to form the PSII reaction center assembly complex. The CtpA1 is recruited to cleave the C-terminal extended portion of pD1 to form the D1 processing intermediate (iD1) which was trimmed to mature D1 (mD1). In the absence of this endopeptidase, neither PSII assembly nor the oxygen evolution activity is operational as the tetra-manganese cluster cannot be formed. This indicates that pD1 processing is necessary for the association of the extrinsic peptides of oxygen-evolving complex toward the luminal side (Selão et al. 2016).

13.4.7 SppA Protease

SppA (*s*ignal *p*eptide *p*eptidase A) is a serine-type protease found in thylakoid membranes of photosynthetic organisms. The SppA family is represented in higher plants by only one enzyme, whereas in most cyanobacteria, SppAs are two proteins (SppA1 and SppA2). SppA1 is a light-induced protease and may be involved in the acclimation of photosynthetic membranes to high light. SppA1 is shown to be involved in the degradation of linker proteins of phycobilisome antennae under acclimation to higher light regimes. About 40% of phycobiliproteins are lost in *Synechocystis* upon irradiance above saturation, and this leads to a bleaching phenomenon (Pojidaeva et al. 2004).

Another high light-induced membrane-integrated protease is SppA2, which is itself a homologue of SppA1 and those of the bacterial SppAs. SppA1 by itself or some Spp1-controlled protease posttranslationally exerts negative control on the cellular pool of SppA2. Interestingly though, SppA1 as a transcriptional activator increased the *sppA2* gene's transcript size. So an increase in *sppA2* mRNA was not accompanied by a concomitant increase in SppA2 protein, but rather a decrease in the net SppA2 level. Due to the high degree of sequence similarity between SppA1 and SppA2 proteins, it is possible that they may share some common functions and, as a result, the decrease in the SppA2 level was compensated by a high light-induced elevated pool of SppA1. Notwithstanding, some slight differences do exist between the actions of SppA1 and SppA2. While SppA1 is involved in photo-acclimation upon high light exposure, the Spp2 was projected to be involved at the recovery stage instead, when already the proteins that were required for photo-protection under high light stress were gathered and are perhaps not required any further (Pojidaeva and Sokolenko 2017).

13.4.8 Cyanophycinase

In *Synechocystis*, an intracellular serine-like exopeptidase with a dimeric structure called cyanophycinase or cyanophycin protease degrades a nitrogen-rich reserve metabolite, multi-L-arginyl-poly-L-aspartic acid (cyanophycin), to supplement nitrogen under nitrogen starvation conditions (Richter et al. 1999; Law et al. 2009).

Arginine is an essential amino acid and can be used as a food supplement. Degradation of cyanophycin by cyanophycinase results in a dipeptide, β-aspartic acid-arginine. Simultaneous production of cyanophycin and cyanophycinase has been an approach to produce the dipeptide in higher plants, viz., *Nicotiana benthamiana*, for which the relevant genes from the cyanobacterium *Thermosynechococcus elongatus* BP-1 were cloned in the plant (Ponndorff et al. 2017). The transgenic plant was a source of the dipeptide, and upon feeding, the dipeptide was transported through murine intestine. However, the availability of free arginine was still a bottleneck, as in the animal system, the dipeptidase that can hydrolyze β-aspartic acid-arginine dipeptide is not present. So the newer approach is to construct a "three-component" transgenic plant, in which cyanophycin synthetase, cyanophycinase and the dipeptidase genes will be simultaneously cloned and expressed in the plastids of forage plants, so that instead of the dipeptide, free arginine from plant-produced cyanophycin can be made available to the animals.

13.4.9 PteB and Other Cell Aggregation Proteases

The term PteB (*p*eptidase *t*ransporter *e*ssential for *b*iofilm) was coined for a cysteine or thiol-peptidase, a gene *Synpcc7942_1133* product of *S. elongatus*, which was required for efficient secretion of a small secreted protein EbfG1-4 with double glycine motif that enables biofilm assemblage formation (Parnasa et al. 2016). Upon insertion inactivation of the gene *Synpcc7942_1133*, release of biofilm protein was hampered, and such mutants exhibited planktonic rather than biofilm growth.

The exoproteome of microbial mats comprising predominantly the filamentous cyanobacterium *Coleofasciculus chthonoplastes* demonstrated the presence of a number of peptidases that include two homogeneous Zn-dependent peptidases, a C-terminal peptidase and D-Ala-D-Ala carboxypeptidases (Stuart et al. 2016). These proteases are believed to be taking part in re-using the extracellular polymeric substances and maintaining the integrity and viability of mats (biofilm).

Several proteases and peptidases were detected that hydrolyze β-casein in the extracellular matrix of *Nostoc punctiforme* PCC70102. These ecto-proteases exhibited sequence similarities with serralysin-like metalloproteases (Vilhauer et al. 2014). Exoproteome analysis in *Anabaena* 7120 also revealed the presence of at least two peptidases, Alr1381 (calcium-dependent peptidase) and All2533 (prolyl endopeptidase), being present in the extracellular matrix (Oliveira et al. 2015). Both rice-field cyanobacteria grow as mats.

The importance of such secretary peptidases is imminent in industry to artificially generate phototrophic biofilms for wastewater purification and bioremediation processes, as also to create efficient biofuel production system with limited water and nutrient inputs.

13.4.10 Orthocaspases: The Prokaryotic Caspase Homologues

Caspases are normally prevalent in the multicellular metazoan organisms and very rarely reported in the prokaryotic world. These classes of cysteine proteases initiate and perform the programmed cell death through several destructive pathways. The first record of occurrence of a homologue of a metazoan caspase was derived from heterologous expression of a *Microcystis aeruginosa* PCC7806 putative caspase-like gene, *MaOC1*. The expressed product was a cysteine endopeptidase that cleaved proteins/peptides with arginine or some basic amino acid in the P1 position (Klemenčič et al. 2015). Due to evolutionary primitiveness of cyanobacterial caspase-like proteases, MAOC1 was referred to as an orthocaspase. Six orthocaspase genes (*MaOC1-6*) were deciphered in *M. aeruginosa*, thus indicating a possibility of several divergent orthocaspases in cyanobacteria. In filamentous cyanobacteria, more number of orthocaspase genes were detected: in *Trichodesmium erythraeum* IMS 101, 12, and in *Anabaena variabilis* ATCC 29413 and *N. punctiforme* PCC70102, 9 each (Klemenčič et al. 2015). In a bloom-forming toxic strain of *Nodularia spumigena*, *the* expression pattern of orthocaspase genes overlapped with concomitant co-expression of the peptide toxin gene of nodularin. This coordinated pattern of expression of the two sets of genes suggests interplay between the toxin biosynthesis and caspase-derived programmed cell death.

In another bloom-forming *M. aeruginosa*, a number of “toxin-antitoxin” genes were found to be located in the vicinity of orthocaspase gene loci, suggesting a close proximity does exists in this organism as well, and toxin production may have some relevance in the bacterial programmed death (Klemenčič and Funk 2018). “Anti-toxin” production should reverse the cell death. Apart from internal factors, environmental deterrent factors, viz., low temperature, darkness, hydrogen peroxide, and polyphenolic allelochemicals, have also been shown to induce caspase-like programmed cell death in *M. aeruginosa* owing to DNA fragmentation (Lu et al. 2017).

13.4.11 PatA and PatG Proteases

Cyanobacteria belonging to the genera *Prochloron*, *Lyngbya*, *Anabaena* (*Sphaerospermopsis*), *Nostoc*, *Microcystis*, and *Trichodesmium* produce a plethora of cyclic peptides which include the ribosomally synthesized posttranslationally modified peptides (RiPPs) collectively grouped under a class, cyanobactins (Sivonen et al. 2010). In the biosynthesis gene clusters, the *patA* and *patG* genes are responsible for cleavage of a pre-peptide to a peptide, which is finally cyclized into

*pat*ellamide *A*, a cyanobactin. The N-C terminal cyclic peptides are posttranslationally synthesized by subtilisin-like serine peptidases Pat A and PatG (Lee et al. 2009). PatA removes the N-terminal motif of the pre-peptide, while PatG recognizes a C-terminal motif to produce an N-C macrocyclized product (Ongpipattanakul and Nair 2018). The post-transcriptional peptidase and macrocyclases are not just restricted to patellamide A alone. As can be seen in *Anabaena* strain 90, the two proteins AcyA and AcyG with homology to PetA and PetG proteolytically degrades a pre-peptide and eventually cyclizes to another cyanobactin, anacyclamide A10 (Leikoski et al. 2010).

Macrocyclization and production of cyclic biologically active peptides is an essential transformation process in nature which can be suitably mimicked in peptide engineering for production of a diverse array of synthetic and semisynthetic hybrid cyclic peptides using the PatG and other enzymes (macrocyclases) (Oueis et al. 2017a, b). Engineered cyclic peptides are sought-after molecules as molecular scaffolds and/or peptide epitopes for drug design because they can mimic the protein's effective domains involved in disease manifestation (Knappe et al. 2011).

13.4.12 Unique Fibrino(geno)lytic Proteases

Recently, three novel serine proteases were discovered from three heterocystous filamentous cyanobacteria: *Anabaena fertilissima* CCC597 (Banerjee et al. 2013), *Westiellopsis ramosa* (Dubey et al. 2018), and *Aliinostoc morphoplasticum* (unpublished work). Interestingly, phylogenetic divergence in the three cyanobacteria did not make much difference in the overall properties of the serine proteases. The unique features such as calcium stimulation but not absolute requirement and inactivation by inhibitors specific for serine proteases and metalloproteases were rarely found in other cyanobacterial proteases, and their N-terminal sequences were also rather unique. We compared the biochemical activity of human plasmin and *A. fertilissima* fibrinolytic protease (Afp) on preformed fibrin (Banerjee et al. 2013). Both of them efficiently liquefied fibrin clot. The degradation pattern of fibrin revealed that, first the γ-dimer was degraded; thereafter, α- and β-chains were partially digested with Afp, whereas plasmin degraded all the three chains. A working model of action is hypothesized and presented in Fig. 13.1. Both proteases (plasmin and Afp) displayed different modes of action and cleaved fibrin differently. Though subject to verification, this attribute of Afp may have a distinct advantage. The plasmin-inhibitory inherent blood factors that keep the plasmin's action under control would not recognize Afp, and consequently, uninterrupted thrombolysis could be expected.

In bacteria, two well-known plasminogen activators, streptokinase and staphylokinase, are considered to be the most effective drugs in thrombolytic therapy. The plasminogen activators indirectly dissolve fibrin clot by converting plasminogen to plasmin. Yada et al. (2005) isolated and characterized an arginine-specific protease, Sp-protease from *Spirulina platensis*, which dissolved fibrin clot, and this activity was enhanced in the presence of plasminogen, suggesting that the

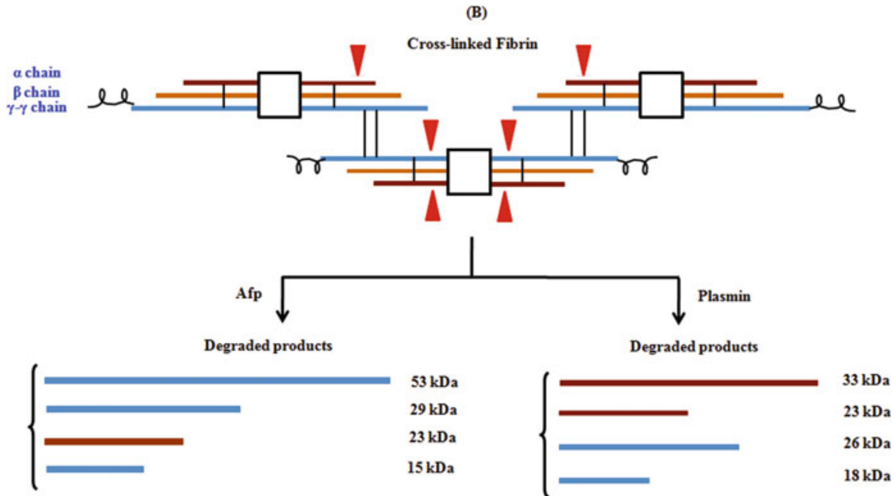

Fig. 13.1 Proposed model showing the comparative action of *A. fertilissima* fibrinolytic protease (Afp) and human plasmin on preformed fibrin clot. Note that the end products (fibrinopeptides) are of different molecular masses

protease acts like a plasminogen activator. Over the period of time, direct clot busters have been isolated from a number of bacterial and fungal sources and few green algae. In this context, the cyanobacterial fibrinolytic proteases have enormous scope for the development of lifesaving drugs that can efficiently dissolve thrombus.

13.4.13 Miscellaneous Proteases

A calcium-stimulated trypsin-like protease was isolated from *A. variabilis* 29,413 (Lockau et al. 1998). A calcium-independent trypsin-like endopeptidase and a prolyl endopeptidase were also partially characterized in *A. variabilis* 29,413 by Strohmeier et al. (1994) and *Anabaena* PCC7120 by Maldener et al. (1991). These enzymes hydrolytically cleaved casein and the synthetic oligopeptide substrates with –Arg and Lys at the P1 position. A subtilisin-like serine peptidase, called subtilase PrcA from *Anabaena* 7120, was heterologously expressed in *E. coli* (Baier et al. 1996).

With the advent of whole-genome sequence available for about 400 cyanobacteria, the putative gene and predicted peptide sequences of a number of proteases are now available in the UniProt database. A number of proteases that have been reviewed and verified by Swiss-Prot are as follows: *Nostoc* 23, *Anabaena* 27, *Synechococcus* 61, *Synechocystis* 48, *Microcystis* 04, *Trichodesmium* 05, and *Cyanothece* 13. Among the proteases listed in Swiss-Prot, Clp, Deg/HtrA, FtsH, HetR, Ctp, and SppA are the most abundant ones. The other prominent proteases in the database are the aminopeptidases, Zn-metalloproteases, and lipoprotein signal peptidases. In this

context, an aminopeptidase-encoding gene, *aapN*, and a zinc peptidase gene, *hap*, were identified in *N. punctiforme* which encode the AapN protein and the zinc protease Hap, both involved in akinete formation (Argueta et al. 2006).

13.5 Conclusion and Future Perspectives

Cyanobacterial proteases are different from the typical degradative proteases of eubacteria and fungi that digest protein and provide nutrition. Instead, the proteases have housekeeping functions to optimally maintain photosystems and nitrogenase complex under dynamic environmental conditions. It should be noted that being phototrophic organisms, the mode of nutrition is photosynthesis and, in some cyanobacteria, nitrogen fixation. These prime nutritional processes are governed, at least in part, by proteases including the chaperons (Heat shock proteins). Thus, they do modulate proteins involved in nutrition. Deg/HtrA, FtsH, SppA, and Clps degrade the damaged D1 protein of PSII due to high irradiation/UV exposure and some peripheral proteins like phycobiliproteins under N starvation state and light-harvesting antenna proteins (IsiA) under iron starvation. Some of them also downregulate PSII core complex proteins, D1 and D2, to deplete oxygen for protecting nitrogenase. On the other hand, Ctp proteases process a pro-protein precursor of D1 into its matured transmembrane form, thereby strengthening PSII assembly with the water-splitting complex. Chaperon/heat shock proteins (Clps, Deg/HtrA) maintain several membranous proteins that are associated with photosynthetic (thylakoid) and cytoplasmic membranes and periplasm. The protein homeostasis facilitates circadian rhythms, cell division cycle, and secretion of unwanted proteins.

Alr3815 shuts down PSII completely for uninterrupted operation of nitrogenase in heterocytes. Additionally, HetR protease, as a transcriptional activator, promotes heterocyte differentiation and spacing, so that nitrogenase is expressed under oxygen-depleted state. Upon N depletion, cyanophycinase provides surplus nitrogen from cellular reserve. Cell aggregation proteases facilitate biofilm formation to protect from grazing, and orthocaspases cause internal apoptosis so that additional healthy cells can replace. A large number of peptides are produced in cyanobacteria and several macrocyclase proteases play a collective role in peptide processing and maturation. These exotic classes of cyclic peptides have varied bioactivities.

Biotechnological applications are perceived of cyano-proteases in preparing antimalarial, antivirulent, and antithrombotic drugs (Clp, FtsH, fibrinolytic), as food additives (cyanophycinase), and in customized synthesis of clinically important exotic peptides (PatA, PatG). More importantly, these proteases make us understand about course of evolution and biogenesis of chloroplasts and photosystem particles in higher plants.

Unfortunately, improper and ever-changing nomenclature and taxonomic benchmarks, on a long-term basis, make it difficult to catalogue the producing

organisms. Even with modern analytical techniques available to characterize the important metabolites with certainty, doubts do exist about the source organisms. Most of the cyanobacteria from which proteases are extracted belong to the orders Synechococcales and Nostocales, both suffering from constant revision and unpredictability. The issues with *Nostocales – Nostoc* and *Anabaena* – have already been addressed; while initially these genera were thought to be monophyletic, subsequent analysis revealed their polyphyletic ancestry with emergence of divergent lineages leading to establishment of several new genera. Species names in many cyanobacteria producing proteases have not been assigned even though partial or complete genome sequences for phylogenetic analysis and habitat and morphological data are available. Modern taxonomists of cyanobacteria must solve all these problems and try to use all the available methodological approaches to arrive at a proper and correct decision for the identification of any strain. Precise analyses of the geographic distribution of various ecotypes and genotypes along with pertinent chemotaxonomic markers depending on the conditions and the strain under study may also act as additional taxonomic markers which could be evaluated as per the case under study.

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14 Microbial Treatment of Waste by Culture-Dependent and Culture-Independent Approaches: Opportunities and Challenges

Juhi Gupta, Bhawna Tyagi, Rashmi Rathour, and Indu Shekhar Thakur

Abstract

Globally, there are concerning trends of waste generation as we progress towards a more developed society. India, the second most populous country, is on the edge of being the fifth largest economy in the world. In the last five decades, its metropolitanization has increased by approximately 10% and would further rise to 20% by 2026. The increasing urbanization and evolving lifestyles, food habits, and standards prompt such exponential expansion of robust waste. It is basically answerable for the introduction of a waste era posing various threats to health and causing ecological deformities. In 1996, the urban number created around 114,576 tonnes of municipal solid waste (MSW) per day, which is predicted to be 440,460 tonnes per day^{-1} by 2026. A large section of the waste is handled using the concept of 3Rs (reduce, reuse, and recycle), but the biological microbial potential is still underestimated. Less than 1% of the microbial community is culturable due to which the conservative cultural microbiology lags behind in revealing the hidden potential. This limitation is subsided by the introduction of culture-independent techniques. This chapter discusses about the possibilities of waste management using microbes both at culture-dependent and culture-independent levels. Next-generation sequencing is a relatively new and flourishing field and promises to be potent enough to supervise the waste generation. Different techniques and procedures have been discussed. The future challenges for waste management lie in the lack of motivation and public unawareness. Waste management is an immediate need of the hour for a sustainable future tomorrow as covered in the Sustainable Development Goals Agenda 2030.

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Solid waste · Microbiology · Microbial community · Culture-dependent approach · Culture-independent approach · Metagenomics · Waste treatment

14.1 Introduction

According to the Resource Conservation and Recovery Act (RCRA) 1976, solid waste is defined as a discarded, abandoned, inherently waste-like, and recyclable material that can be a solid, semi-solid, liquid, or contained gaseous material. It can be any garbage, refuse, and discard from industrial, mining, municipal, and agricultural operations. According to a report from World Bank (2012), the municipal solid waste (MSW) is generated globally at a rate of 1.3 billion tonnes per year, and the average MSW generation rate was 1.2 kg/capita/day. It is expected that by 2025, the global MSW generation rate will be 2.2 billion per year and by 2050 will be 4.2 billion per year. Generally, a linear relationship has been seen between GDP/capita and MSW generation rate/capita in most of the countries (Kumar and Samadder 2017). For developed countries, waste generation rate is from 1 to 2.5 kg/capita/day, and for developing countries, it is from 0.50 to 1 kg/capita/day. The possible reasons for high MSW generation rate are population explosion, uncontrolled and rapid industrialization, and improved living standards due to rapid urbanization. The major portion of the total generated solid waste is dumped inefficiently and without proper waste management at outskirts of towns or cities. Agricultural waste is also disposed without any pretreatment, and industries are also dumping their wastes illegally without prior treatment (Nandan et al. 2017).

The management of municipal solid waste is a major environmental problem in Indian megacities because of lack of regulation of proper MSWM methods (Sharholy et al. 2008). It is seen that in India, solid waste collection efficiency is around 70% in comparison with other developed countries (Sharholy et al. 2007), which shows the inefficiency of municipal solid waste management system in India. Inefficient municipal solid waste management in India leads to increase in various environmental problems like air pollution due to release of gases from waste, groundwater pollution due to leachate percolation, etc. Therefore, there is a need for implementation of effective solid waste management in India (Nandan et al. 2017).

14.2 Overview of Solid Waste

14.2.1 Different Types of Waste and Its Upsurge

There are three types of classification of solid waste according to (a) origin of solid waste, (b) its contents, and (c) its potential hazard. Classifying the waste using different categories will aid us in realizing its true nature and further segregating it with maximum efficiency for better waste management.

14.2.1.1 Categorization of Solid Wastes According to Their Origin (Tchobanoglous et al. 1977)

(a) *Residential* – This is composed of wastes generated from buildings and apartments, i.e., leftover food, vegetable peels, plastic, clothes, wood, glass, metals, electronic items, ashes, etc.
(b) *Municipal* – This consists of wastes generated from street cleaning, landscaping, parks, waste treatment plants, tree trimming, sludge, and other recreational areas.
(c) *Industrial* – This refers to wastes from industrial activities and consists of process wastes, ashes, demolition, and construction wastes.
(d) *Commercial* – This includes wastes from stores, restaurants, markets, hotels, medical facilities, and auto repair shops (leftover food, glass, metals, ashes).
(e) *Constructional* – This refers to wastes generated from constructional activities like road repair, demolition of old buildings, and construction of new buildings which include metal, plastic, glass, cement, rocks, electrical wastes, etc.
(f) *Agricultural* – This refers to wastes generated from fields, farms, crops, dairies, and vineyards, which include spoiled food grains and vegetables, agricultural remains, litter and hazardous waste like pesticides, etc.
(g) *Institutional* – This refers to wastes generated from educational, administrative, and public buildings like schools, colleges, and offices, which include paper, glass, plastic, metal, leftover food, etc.
(h) *Biomedical waste* – This refers to wastes from hospitals, medical shops, research laboratories, and clinics, which include gloves, bandages, disposable syringes, instruments, chemicals, blood and body fluids, sanitary materials, and blood-containing textiles.
(i) *Mining waste* – This refers to wastes generated from open-cast mining and underground mining, which include inert materials such as ash.

14.2.1.2 Categorization of Solid Wastes According to Type of Contents Present (Phelps 1995)

(a) *Garbage* – These are wastes of animal and plant origin generated from handling, serving, storage, cooking, etc. It contains mostly putrescible and easily decomposable organic matter.
(b) *Ash and residues* – This waste category comprises substances remaining after burning of combustible materials such as coal and charcoal coke used for cooking and heating purposes in houses, industries, and institutes.
(c) *Combustible and noncombustible* – Combustible wastes are organic components of solid waste that can burn like paper, cardboard, wood, textiles, rubber, leaves, household waste, and plastic. Noncombustible wastes include glass and metals, which are not organic in nature and cannot burn.
(d) *Biodegradable and nonbiodegradable* – Solid waste which is organic in nature and can be degraded by microorganisms in a short period of time is called biodegradable waste, for example, paper, food, wood, etc., while waste which cannot be degraded by microorganisms is called nonbiodegradable such as plastic, metal, and glass.

(e) *Bulky waste* – This refers to large-sized waste from households such as tires, electrical appliances (refrigerator, washing machine, etc.), furniture, vehicle parts, wood, trees, branches, etc.
(f) *Street waste* – These wastes include paper, dirt, cardboard, food matter, plastics, and leaves collected from streets, parks, and walkways.

14.2.1.3 Categorization of Solid Waste According to Hazardous Potential

(a) *Hazardous* – Under the RCRA (Resource Conservation and Recovery Act) 1976, hazardous waste is defined as solid waste that exhibits at least one of the following characteristic properties: ignitability, corrosivity, reactivity, or toxicity. These are generated from manufacturing, industrial processes, and commercial products.
(b) *Nonhazardous* – Solid wastes that do not show properties of toxicity, corrosivity, ignitability, and reactivity are considered as nonhazardous.
(c) *Radioactive* – It refers to waste generated from nuclear power reactors, research laboratories, hospital, and foul processing plants.
(d) *Infectious* – This refers to medical wastes which have potential to transmit viral, bacterial, fungal, or parasitic diseases. It includes both human and animal infectious waste and waste generated from pathological laboratories, research laboratories, and veterinary services (Singhal et al. 2017).

14.2.2 Present Scenario

According to data from the 2011 Census of India, India has an overall population of about 1.21 billion, i.e., 17.5% of the total world population. Out of the total population of India, about 64% are rural and 32% are urban populations (World Bank 2014). Out of the 475 urban agglomerations of India, five are heavily populated: Mumbai having 18.4 million, Delhi 16.3 million, Kolkata 14.1 million, Chennai 8.7 million and Bangalore 8.5 million population (Census of India 2011). Due to rapid and increased urbanization and improved lifestyle, it is seen that cities in India are generating more MSW in comparison with the total MSW generation of 1947 (Table 14.1) (Sharholy et al. 2008). Metro cities in India are generating more MSW than small towns and rural areas (Table 14.2) (Dayal 1994; Kansal 2002; Singh and Singh 1998).

It is reported that MSW quantity (tonnes/day) and its per capita generation rate are elevated in states with high income (per capita). For example, Delhi shows such rate elevation due to the improved high living standards, rapid growth of income, and rapid urbanization. These parameters are currently under control for medium-income states (per capita) like Himachal Pradesh, Gujarat, etc. and low-income (per capita) states like Uttar Pradesh, Bihar, Manipur, Madhya Pradesh, etc. (Kaushal et al. 2012).

Table 14.1 Waste generation per capita in Indian cities

Population size	Number of cities surveyed	Waste generation (kg/capita/day)	References
<0.1 million	8	0.17–0.54	Kumar and Goel (2009) and Kumar et al. (2009)
0.1–0.5 million	11	0.22–0.59	
1–2 million	16	0.19–0.53	
>2 million	13	0.22–0.62	

Table 14.2 Per capita MSW generation data (2010–2011) of megacities in India

City	Population (2011) $\times 10^6$	Total waste generated (tonnes/day)	Waste generation (kg/capita/day)
Ahmedabad	6.3	2300	0.36
Hyderabad	7.7	4200	0.54
Bangalore	8.4	3700	0.44
Chennai	8.6	4500	5.2
Kolkata	14.1	3670	0.26
Delhi	16.3	5800	0.41
Mumbai	18.4	6500	0.35

Source: Status of compliance by CPCB with municipal solid wastes (management and Handling) rules, 2000 (CPCB 2012)

In Indian cities, among the overall compositional categories of MSW, about 41 wt.% are organic, 40 wt.% are inert, and 19 wt.% are recyclable materials (Sharholy et al. 2008). The difference in the MSW characteristics tells about the urbanization and developmental effects in a region. Out of the total MSW, the relative percentage of organic waste present generally increases as the socioeconomic status decreases; therefore, rural areas of India generate a high amount of organic waste than urban areas. Among the total generated MSW in urban areas, compostable components constitute 40–60% and inert materials 30–50% (Sharholy et al. 2008).

14.2.3 Source

Sources of solid waste include residential sites (apartments, buildings), municipal sources (street, waste water treatment plant, parks), construction sites (demolition of existing structures, construction of new buildings, and road repair), industrial sites (electricity generation plants, processing industries, and manufacturing units), commercial sites (hotels, office buildings, restaurants, hospitals, and markets), institutional sites (colleges, schools, universities, etc.), and agricultural sources (farm, vineyard, crop field, etc.) (Nandan et al. 2017). Detailed information about the sources of solid waste is discussed earlier.

14.2.4 Composition and Characteristic Features of Wastes

In India, the major compositional categories of solid wastes are the following (Joshi and Ahmed 2016):

(a) *Biodegradable waste* – This includes waste from the kitchen (food), plants (fruits, leaves, flowers, and vegetables), paper, and wood.
(b) *Composite waste* – This includes waste clothes and plastic waste such as toys and tetra packs.
(c) *Household hazardous and toxic waste* – This includes medicinal, electrical, and electronic wastes (such as fluorescent tubes and light bulbs), chemicals, paints, batteries, fertilizer, pesticide container, and shoe polishes.
(d) *Recyclable waste* – This includes paper, glass, cardboard, bottles, cans, metals, and certain types of plastics.
(e) *Inert waste* – This includes dirt, debris, and C&D.

The characteristic features of wastes in Indian cities according to the population size are given in Table 14.3 (physical characteristics) and Table 14.4 (chemical characteristics).

Table 14.3 Physical characteristics of MSW in Indian cities (population wise)

Population size (in millions)	No. of cities surveyed	Paper	Rubber, leather, and synthetics	Glass	Metal	Compostable matter	Inert material
0.1–0.5	12	2.91	0.78	0.56	0.33	44.57	43.59
0.5–1.0	15	2.95	0.73	0.56	0.32	40.04	48.38
1.0–2.0	9	4.71	0.71	0.46	0.49	38.95	44.73
2.0–5.0	3	3.18	0.48	0.48	0.59	56.57	49.07
>5.0	4	6.43	0.28	0.28	0.8	30.84	53.9

Source: NEERI report strategy paper on SWM in India, August 1995 (all values are expressed in percentage)

Table 14.4 Chemical characteristics (in %) of MSW in Indian cities (population wise)

Population size (in millions)	Nitrogen as total nitrogen	Phosphorus as P_2O_5	Potassium as K_2O	C/N ratio	Calorific value, kcal/kg
0.1–0.5	0.71	0.63	0.83	30.94	1009.89
0.5–1.0	0.66	0.56	0.69	21.13	900.61
1.0–2.0	0.64	0.82	0.72	23.68	980.05
2.0–5.0	0.56	0.69	0.78	22.45	907.18
>5.0	0.56	0.52	0.52	30.11	800.7

Source: NEERI report strategy paper on SWM in India, August 1995

14.2.5 Sink

SWM system comprises of elements such as waste collection, storage, transport, and disposal, and in a country like India, this effective SWM is a major challenge (Kumar et al. 2017). In India, the Municipal Corporation is responsible for waste collection. Although there are separate bins for biodegradable and inert materials, due to lack of proper and effective management, mixed waste (i.e., biodegradable and inert waste) is often dumped and burnt openly (Kumar et al. 2017). In India, segregation of MSW (biodegradable waste, nonbiodegradable waste, e-waste, and toxic waste) is not done in an organized and scientific way at both levels, i.e., household and community bins (Joshi and Ahmed 2016). It is believed that approximately more than 90% of the generated MSW in India is dumped in an unsatisfactory way (Kumar et al. 2017), i.e., the waste is dumped without proper segregation of biodegradable and inert components of waste in community bins where optimal recycling is not done. It is seen that rag pickers mostly sort, extract, and sell out recyclable materials like plastics and glass from dumpsites, waste bins, trucks, and streets (Joshi and Ahmed 2016).

MSW transportation is usually done through trucks, hand rickshaw, bullock cart, compactor, tractors, tanker, trailers, and dumpers in Indian cities and towns. Only a small percent of waste is transported through covered vehicles (Joshi and Ahmed 2016). According to CPCB, in India, 50% of MSW are collected manually, while 49% are through trucks (CPCB 2000a, b, c). Now in some megacities (Delhi, Mumbai, Madras, Hyderabad, and Bangalore), door-to-door solid waste collection has been started. This is due to the difficulty in collection and disposal in low-income states because of unwillingness of the people to pay for collection service (Kaushal et al. 2012). In a few of the Indian cities and states, the average collection efficiency for the total generated MSW is about 72% which shows the high collection and transportation efficiency of the total generated MSW in that states where private contractors and NGOs are employed (Kaushal et al. 2012).

Solid waste disposal in India is suffering from lack of resource and techniques. Various new techniques and alternatives have been proposed, including the use of optical sensors and GIS (Geographic Information System) technology, but these are not in proper use currently. In India, there is an urgent need for properly engineered waste disposal which can protect public health and environment and its resources such as groundwater, surface water, soil fertility, and air quality (Kumar et al. 2017).

14.2.6 Transformation

Once solid waste is collected, segregated, and transported, it is dumped at different open dumping or landfill sites. At such sites, the organic component of solid waste is decomposed by small insects and further degraded by microbes aerobically and anaerobically (bacteria, fungi, algae, etc.). Landfill releases some harmful gases like methane (major contribution, i.e., 50%), carbon dioxide, hydrogen, and others from decomposition of biodegradable waste under anaerobic condition

(Kaushal et al. 2012). Dumping harmful chemicals and antibiotics is a major threat because over time, these chemicals convert to other harmful and recalcitrant compounds which are even more difficult to remove. Inert wastes like plastic, metal, glass, and cardboard are sorted from the decomposable waste and are recycled into useful materials. Biomedical wastes from hospitals are incinerated in low-oxygen condition mostly, and energy is produced from it. Some of the organic contents of solid waste are transformed into energy and recoverable material by thermal treatment methods such as pyrolysis, combustion, and gasification. Detailed information about the physical, chemical, and biological transformation of solid waste is discussed further.

14.2.7 Problems Related to Increasing Waste (Locally, Regionally, and Globally)

It is observed that the impacts of inefficient and inadequate solid waste management are not limited to local and regional level but are crossing the boundaries and affecting the global environment (e.g., methane emission is causing global warming) (Kaushal et al. 2012). In low- and middle-income developing countries, the major fraction of MSW is dumped mostly in slum and low-lying areas. It is observed that due to lack of strict regulations, potentially infectious, medical, and hazardous waste get mixed up with MSW that is very harmful to rag pickers, people around that site, and the environment there. Most of the water bodies (groundwater and surface water) are getting contaminated due to migration of leachate from open dumps or landfills, industrial wastewater, agricultural wastewater, etc. (Kaushal et al. 2012; Swati et al. 2018). There are well-known impacts of inadequate waste management on public health such as increasing cases of throat and nose infections, problems in breathing, bacterial infection, allergies, asthma, reduced immunity, anemia, inflammation of the lungs, and other infections (Khan and Burney 1989). Every year in Mumbai, open burning of MSW emits about 22,000 tonnes of pollutants into the atmosphere (Dayal 1994).The cases of increasing smog problems and respiratory diseases are emerging because of the release of fine particles due to uncontrolled burning of waste at dump sites (CPCB 2004).

Open waste dumps have many adverse impacts on human health, flora, fauna, and the environment. Landfill releases some harmful gases like methane (major contribution, i.e., 50%), carbon dioxide, hydrogen, and others from decomposition of biodegradable waste under anaerobic condition (Kaushal et al. 2012). Other problems are odor coming out from open dumps, leachate migration from dumps to water bodies, and increasing waterborne diseases (malaria, dengue, etc.). This happens due to breeding of insects in stored water in discarded materials like tires and bottles at open landfill sites (Kaushal et al. 2012). Global warming due to increased GHG emissions from MSW has emerged as a major concern. It is seen that landfill solely contributes 50% of total global methane emissions which is mostly contributed from municipal solid waste sector (IPCC 2007). MSW was the third largest anthropogenic source of methane gas, i.e., around 3–4% of global

anthropogenic gas emissions (Annepu 2012). The total waste sector contributed 18% of global methane emissions (Kumar et al. 2017).

14.3 Possible Solutions to Deal with Increasing Waste

Solid waste is transformed physically, chemically, and biologically to reduce the weight and volume of waste and to recover energy, conversion products, and reusable and recyclable materials from it. This helps in increasing the efficiency of solid waste management systems.

14.3.1 Physical Treatment

In this treatment method, the major focus is on changing the phase of solid waste. The following methods are used to reduce the volume and to recover conversion product from MSW.

14.3.1.1 Land Filling

It is reported that in India, 59 landfill sites are fully constructed and working and 376 landfill sites are under planning and inception stage (CPCB 2013). In India, landfilling is the most common and extensively practiced dumping option. It is observed that in some of the metropolitan cities (Delhi, Mumbai, Kolkata, Chennai), most of the landfill sites are running out of their waste-holding capacity because of limited availability of space for waste disposal (Sharholy et al. 2008).

14.3.1.2 Open Dumping

Disposal of municipal solid waste directly on open low-lying areas is called open dumping. The major fraction of municipal solid waste in India is illegally dumped at such sites by common people. Open dumping causes various environmental problems such as air pollution and groundwater pollution.

14.3.1.3 Recycling

In this method, recyclable materials like paper, glass, plastic, rubber, cardboard, and metal from the solid waste are recovered, reused, and recycled. Rag pickers play an important and major role in the recycling process, as they work whole day in collecting and recovering the potential recyclable material from sites such as roads, community dustbins, and disposal sites (Sharholy et al. 2008). The global recovery rate of paper waste was 37%, while in India, paper waste recovery was about 14% of the total consumption of paper in the year 1991 (Pappu et al. 2007). In India, 40–80% fraction of plastic waste is recycled annually, but only 10–15% of plastic waste is recycled in developed countries (Sharholy et al. 2008).

14.3.2 Chemical Treatment

In this treatment method, the aim is to change the phase of waste and recovery of energy. The following methods are used in chemical treatment.

14.3.2.1 Gasification

It is the combustion of organic contents of solid waste in conditions such as low or absence of oxygen at high temperatures. The main aim of gasification process is the production of syngas from which energy can be produced by combustion. Further, it could be utilized to produce feedstock for chemical and liquid fuel (Kumar et al. 2017). In India, there are two types of gasifiers designs for the treatment of agro waste, sawmill dust, and forest waste. The first design gasifier is established at Nohar and Hanumangarh, Rajasthan, while the second one is at TERI, Delhi (Ahsan 1999).

14.3.2.2 Pyrolysis

It is the combustion of solid waste in the absence of oxygen at a temperature condition of about 400–800 °C. The products of the pyrolysis process at low temperature range (500–550 °C) are gas, oil, and tar, and at high temperature (>700 °C), gases (methane, carbon dioxide, carbon monoxide, and hydrogen) are the main products (Kumar et al. 2017). It is reported that good-quality pyrolysis products are formed when the feedstock, i.e., solid wastes, is of specific type such as plastic, electric, electronic waste, wood waste, etc. (Lombardi et al. 2015).

14.3.2.3 Incineration

In the incineration process, solid waste is completely combusted in a controlled manner. The temperature range for the incineration process is 980–2000 °C. Almost 80–90% of combustible solid waste present in MSW can be reduced by this method (Sharholy et al. 2008). In India, incineration of MSW is a bit difficult task because of the high-range requirement for each (30–60%) and every organic constituent, moisture constituent, or inert content in waste. The caloric value is in the range of about 800–1100 kcal/kg of MSW (Joshi and Ahmed 2016). This process is mostly used for treating hospital waste in India (Sharholy et al. 2005). In India, there is no large-scale functional incinerator for MSW.

14.3.2.4 Refuse-Derived Fuel (RDF)

The generation of fuel for energy using the discarded waste is called refuse-derived fuel. As RDF plant produces improved solid fuel or pellets from MSW, we can say that RDF plant helps in reducing the pressure on landfills (Joshi and Ahmed 2016). The establishment cost for RDF plant is very high due to which very few RDF plants are set up in India, for example, at Hyderabad, Guntur, and Vijayawada in Andhra Pradesh.

14.3.3 Biological Treatment

In this treatment method, the major focus is on reducing the volume and weight and also the production of useful materials using microorganisms such as biodiesel from sludge, compost formation, and gases like methane, etc. (Kumar et al. 2016).

Microorganisms are an undiscovered and underutilized asset for bioremediation. Naturally, the original cycling in nature is maintained by microbes primarily bacteria and fungi. They possess the capability to transform the natural and artificial chemicals into raw materials and energy sources for maintaining their vitality. Thus, they are a cost-effective, environment-friendly, and better alternative to the expensive physical or chemical remediation processes. Microbial remediation is a reliable and simple method with many advantages in terms of biodegradable matter and nitrogen compounds removal (Medhi and Thakur 2018). Biological treatment of solid waste involves the following.

14.3.3.1 Aerobic Digestion

This process involves the digestion of organic matter present in waste under a humid and warm environment in the presence of oxygen/air (Joshi and Ahmed 2016). In India, many composting units are present in locations like Indore, Bengaluru, Vadodara, Mumbai, Delhi, and Kanpur, with an average installed capacity of 150–300 tonnes/day (Sharholy et al. 2007). Aerobic treatment includes activated sludge, well-aerated lagoons, and aerobic biological reactors.

14.3.3.2 Anaerobic Digestion/Biomethanation Process

Biomethanation is the process involving anaerobic digestion of organic matter present in waste in the absence of air or limited air, and biogas is the product of this process. There are many other anaerobic microbial processes which are employed in the treatment of solid waste like anaerobic filter and lagoon, fluidized bed, upflow anaerobic sludge blanket (UASB), and anaerobic contact reactors. In India, biomethanation-related schemes are under planning and implementation stage in Delhi, Bangalore, and Lucknow (Ambulkar and Shekdar 2004). Methane gas (around 55–60%) present in biogas which is generated during anaerobic digestion is used as a fuel for various purposes like power generation, cooking, etc.

14.3.3.3 Vermicomposting

In vermicomposting, semi-decomposed organic waste (first it is decomposed through microbes) is treated by introducing earthworm on it. In India, the main vermicomposting plants are located in Hyderabad, Bangalore, Faridabad, and Mumbai (Joshi and Ahmed 2016). Biological treatment of waste is elaborated in detail in the next section with a major focus on culture-dependent and culture-independent methods of microbiology.

14.4 Why Study Microbiome?

Unlike the research conducted on flora and fauna communities, the microbial world still lacks the deserved recognition. The microbes available in various ecological samples are very efficient repositories of many important metabolic functions. Their efficiencies and capabilities are still underestimated. As more than 99% microbes are noncultivable, we prefer to unveil them using the new sequencing technologies like metagenomics (Wobus et al. 2003). Microbes are the maintainers of the biogeochemical cycles, fertility of each space, and even a part of many useful symbiotic relationships which are important for existence (Fierer et al. 2012). These rich inventories of our environment are fairly influenced by many parameters like pH, temperature, moisture, carbon input, nutrient availability, etc. (Fierer et al. 2007; Goldfarb et al. 2011). Such microbial studies at culture-dependent and culture-independent levels are continuously expanding in order to reach the deeper depths of taxonomic and functional metabolism.

14.5 Microbiology in Waste Management

Earth has provided us with everything having its own replenishing system. From the beginning of life, various ecological processes involving physical, chemical, and biological changes are maintaining an inherent stability. But with changing industrialization and urbanization, it is losing its self-maintenance capacity due to the formation of various toxic and recalcitrant compounds. Generation of solid waste has become a challenging problem for the survival of the human race as well as environment. Waste sinking in landfills is attracting global concern today (Gupta and Mohapatra 2003). With rapid civilization, 1000 new chemicals (approximately) are added to the existing list every year. Citing the third world report, about 460 million kilograms of pollutants are released worldwide in the environment (Singh and Tiwari 2014). Nationally and internationally, many researchers are involved in overcoming this problem of solid waste menace (Singh and Tiwari 2014).

Microbiological processes have been always underestimated with respect to their significance in waste management and are even very less exploited (Gandolla and Aragno 1992). Today, the amalgamation of technology with biology will aid us with a solution. Most of the wastes end up in landfill as this seems the easiest and accessible option. Landfill can be thought of as a big anaerobic bioreactor where the combination of many useful microbes helps in waste processing to ultimately release methane and carbon dioxide (Gandolla and Aragno 1992). Using the helpful interactions of the available microbial consortia, we can optimize the waste management. Initially, it should be dealt by conducting pilot-scale experiments in the laboratory and then commercializing the research in the real field.

Remediation using the potential of microbes is an environment-friendly and cost-effective biological alternative to conservative methods of physical and chemical waste management (Singh and Tiwari 2014). Microorganisms help in dealing with many problems like hazardous waste, heavy metal removal from contamination

sites, purifying the polluted air, etc. If the potential of the microbes would be explored in the right direction, dealing with the enormous amount of waste would become more feasible.

Examples of Biological Management of a Waste Site

1. Post-collection, the organic fraction of the waste should be entertained properly to increase the production of quality biogas. Better-quality biogas will reduce other harmful emissions.
2. The quality of fluid released will also improve which will increase the overall stability of a landfill site.
3. Biological management will allow us to avoid/decrease chemical treatment having many worse limitations.
4. Emissions of uncomforting odors can be controlled using biofilters.
5. Organic waste with low amount of lignin content can be processed using biomethanization.
6. Efficient category of strains should be isolated using laboratory microbiological techniques which should be exploited further in waste treatment.
7. Medical waste should be treated discretely. Although substances like antibiotics are biodegradable, they can hamper the growth of some nonresistant useful bacteria.
8. Composting should be taken as a serious and compulsory alternative.

With the upcoming technological advancements, many amendments are done in the remediation strategies to increase the efficiency rate of microbes like:

(a) Biosurfactant-producing isolates to increase the working area for the microbe
(b) Bioremediation in the targeted rhizosphere (Kumari et al. 2016; Sharma et al. 2017)
(c) Introduction of genetically modified or transgenic microbes

Several enzymatic complexes are involved in remediation activities like monooxygenases, dioxygenases, laccase, peroxidase, and others. A few of the examples with applications are given in Table 14.5.

Table 14.5 Examples of microbial enzymes and activity involved in degradation

Enzyme	Activity	Application
Monooxygenase and dioxygenases	Oxidation of substrate by transfer of oxygen	Detoxification of harmful compounds like phenols, chlorinated organics, etc.
Peroxidases	Substrate oxidation involving H_2O_2	Phenolic compounds, lignin
Lipases	Degradation of hydrocarbon	Bioremediation of common waste sites
Hydrolytic enzymes	Disruption of chemical bonds	Decreases the compound activity, making it harmless

Source: Kumari et al. (2017)

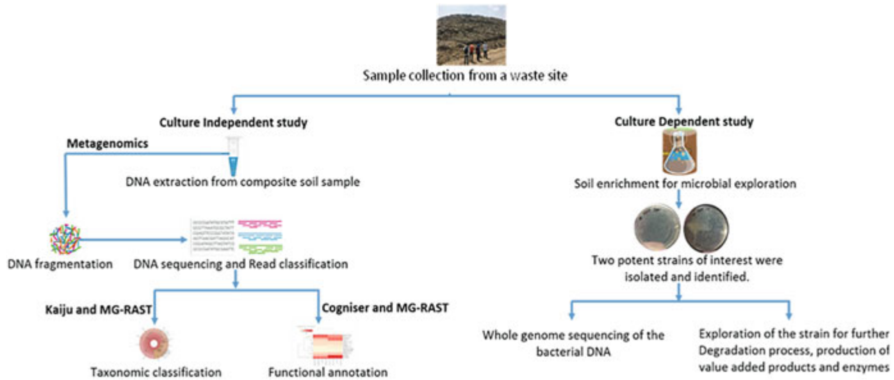

Fig. 14.1 Culture-dependent and culture-independent techniques to study microbial diversity of a site

14.5.1 Approaches in Microbiology

In microbiology, we follow two different approaches while achieving a specific target (Fig. 14.1 briefly demonstrates both culture-dependent and -independent techniques while studying the microbial diversity of a particular site).

Culture-Dependent Approach In this technique, we culture microbes in a nutrient media and process the culture further with a specific aim. This involves the hands-on experimentation using microbes in a working space. The microbial potential in terms of degradation of toxic compounds, production of by-products, etc. is also explored.

Culture-Independent Approach An evident fact that >99% microbes are unculturable gave rise to the discovery of culture-independent approach like next-generation sequencing. There are different techniques involved in it like metagenomics, metabolomics, etc. In this, we work over the DNA or RNA sequences to completely align their nucleotides. This is matched up with a reference database, and after computational analysis, the taxonomic and functional characterization is done. Below is a detailed discussion for both approaches.

14.5.1.1 Culture-Dependent Approach

There are two categories of bioremediation:

(a) *In situ remediation.* It involves treatment at the polluted site itself without involving any excavation and transport of contaminants. In this, the site-specific indigenous microbial flora plays an important part (Agarwal 1998). This remediation is cost-friendly and widely acceptable (Singh and Tiwari 2014). Methods like biosparging, bioventing, and bio-augmentation are usually used (as discussed in Table 14.6). This method is favorable for less polluted sites.

Table 14.6 Examples of different techniques involved in in situ and ex situ bioremediation with applications

Name of technique	Application	Removal	Conditions	References
In situ bioremediation				
Biosparging	Injection of air under pressure below the water table. This would increase the concentration of oxygen, aiding the process of degradation by the indigenous microbial flora	Gasoline, diesel and jet fuel	Very efficient in areas with permeable soil Easy installation and short treatment time	Bouwer and Zehnder (1993)
Bioventing	Efficiently removes any aerobically degradable compound The main process involves the injection of oxygen and different nutrients like phosphorus, nitrogen, etc., in areas which are short of air	Petroleum, oil, gasoline	In areas with deep water tables and high temperature, the efficiency of the process increases	Niu et al. (2009)
Bioaugmentation	Microbes capable of degrading or remedying the pollutants to form less toxic or nontoxic products are used to enhance the process of in-situ bioremediation Many different case studies are available	Organic pollutants (chlorinated, nitrated, etc.) from soil and wastewater	Involves natural attenuation in bioreactors Both native microbes and augmenters are utilized	Stephenson and Stephenson (1992), Qasim and Stinehelfer (1982) and Bouchez et al. (2000)
Ex situ bioremediation				
Landforming	This involves a sandwich treatment of the contaminated soil between a fresh	Pesticides	Heat, oxygen, nutrient, and pH (7) balance is important	Antizar-Ladislao et al. (2008)

(continued)

Table 14.6 (continued)

Name of technique	Application	Removal	Conditions	References
	soil and a concrete layer			
	Natural degradation is augmented		Cost-effective process	
Composting	This involves the usage of microbial potential	Organic waste; lignin, cellulose, hemicellulose	Maintenance of 75% moisture is required.	Tuomela et al. (2000), Sjostrom (1993) and Makan et al. (2013)
	While performing the metabolic activities, microbes elevate the ambient temperature which helps in solubilizing waste and degrade it further		Involves enzymes like laccase, peroxidase, etc.	
Biopiling	This technique is a combination of land farming and composting	Helpful for pesticides	Control of heat, moisture, oxygen, nutrients, and pH is important at the treatment site	Tiwari and Singh (2016)
	A treatment bed with an irrigation/nutrient system and proper aeration is utilized			

(b) *Ex situ remediation.* When the remediation is carried out at a different place from the contaminated site, it is termed as ex situ remediation (Singh and Tiwari 2014). Transportation increases its procedural cost, but it gives many effective results in decreasing the load of toxic xenobiotic compounds (Kumari et al. 2014). There are different possibilities for ex situ bioremediation like biopiling, landforming, bioreactors, etc. (Table 14.6).

Nationally and internationally, many researchers are involved in studying the microbial degradation of toxic compounds at the laboratory scale. The following paragraph will brief one of the case researches, discussing about one of the many potential microbial isolates used in remediation.

In this study, Kumari et al. (2014) discussed about the capability of a microbial strain *Paenibacillus* sp. ISTP10 isolated from sludge waste to degrade the toxic endosulfan. A soil microcosm was set up spiked with a known concentration of the toxic compound to test the isolate. Over the time, different samples were collected

Table 14.7 Bioremediation of heavy metals, agricultural waste, and rubber waste

Contaminant	Activity	References
Heavy metals	Due to their toxicity, heavy metals are a matter of concern as they not only pollute terrestrially but also pollute the aquatic sources by metal leaching.	Jayashree et al. (2012) and Chen et al. (2008)
	Sequestration of heavy metals is done by the interaction between the positively charged metal ion and the negatively charged microbial membrane. It is then transported and bioaccumulated	
	Example: *Cupriavidus* sp., *Pseudomonas aeruginosa*	
Agricultural waste	A large part of waste comes from the agricultural sector. To deal with this waste, the best technique is vermicomposting in which we use a combination of earthworm and microorganisms.	Garg et al. (2006)
	Earthworm species like *Eisenia fetida* are involved	
Rubber waste	It constitutes 13–16% of generated solid waste. The major harmful constituents of rubber when burned will lead to generation of toxic fumes and hamper the survival of healthy microbes.	Bredberg et al. (2001)
	Oxidizing bacteria like *Pyrococcus furiosus* are helpful in degradation of rubber	

for their GC-MS (Gas chromatography-mass spectrophotometry) analysis. The analysis showed the presence of intermediate degraded metabolites of endosulfan. The study even analyzed the toxicity of the degraded metabolites. The strain proved to be efficient enough for bioremediation studies of contaminated sites. Such pilot-scale experiments at lab can be extended to the real practical fields in order to augment the regular process of microbial remediation.

There are many microorganisms which are specifically used for targeted waste like heavy metals, agricultural waste, etc. Table 14.7 lists the activity involved in degradation of specific waste material using efficient microbes.

According to the three-domain concept of microbial classification system, microbes of interest are present in *Bacteria*, *Archaea*, and Eukarya. Due to their physiological adaptability and metabolic efficiency, they are capable of degrading a wide range of compounds. Table 14.8 lists the important groups.

As discussed in the above sections, the culture-dependent method is useful and evolving with the upcoming advancements. It comes with some advantages as well as limitations. Currently, a large section of microorganisms is unculturable, for example, *Acidobacteria*; this will restrict us to reveal the true potential of microbial flora. For this, we seek the aid from culture-independent technologies.

14.5.1.2 Culture-Independent Approach

This involves the genomic analysis of all the community nucleotides like DNA, RNA, etc. using sequencing techniques. As mentioned earlier, this involves different methods like metagenomics (involves processing of whole DNA sample from a particular environment), metatranscriptomics (involves processing of RNA from an

Table 14.8 Important taxa with their activity involved in bioremediation

Organism	Activity	Enzymes involved and examples	References
Bacteria	Exhibit different catabolic pathways which are efficient enough to degrade a large array of harmful compounds like furans, dioxins, PAH (polycyclic aromatic hydrocarbons), excess nutrients, etc. in different environmental samples	Hydroxylases, monooxygenase, dioxygenase, etc.	Ali et al. (2014), Karigar and Rao (2011) and Singh et al. (2014)
		Pseudomonas, *Cupriavidus*, *Shewanella*, *Paenibacillus*, *Bacillus*, *Pandoraea*, etc.	
Fungi	This class of remediation is called as mycoremediation (using fungi)	Phase I and II enzymes like Cytochrome P450 monooxygenase (P450s) and epoxide hydrolases are involved	Gielkens et al. (1997)
	Fungi use their extensive hyphae for quick colonization to degrade the pollutants like lignin, phenolic compounds, etc.	*Aspergillus niger*, *Aspergillus tubingensis*, *Fusarium ventricosum*, *C. elegans*, etc.	
Algae	This section has been highly underestimated regarding their potential in bioremediation	Antioxidant defense system with catalase and superoxide dismutase (SOD) takes part in the process	Karigar and Rao (2011), Zhang et al. (2012), Mishra et al. (2018a, b) and Sethunathan et al. (2004)
	They are highly efficient in degrading herbicides, some PAHs, and insecticides	*Anabaena azotica*, *Chlorella*, *Chlamydomonas* sp., etc.	

ecosystem sample), whole-genome sequencing (processing of DNA from a single and pure isolate), whole-transcriptome sequencing (processing of RNA from a single and pure isolate), etc. Soil, water, sediments, and other environmental samples are rich sources of microbial diversity (Rathour et al. 2017). Next-generation sequencing would help us to overcome the cultural limitations and unravel the hidden microbial potential both taxonomically and functionally. To understand this further, below is the description for one of the techniques, metagenomics.

A. **Metagenomics**

Metagenomics is defined as the culture-independent genomic analysis of microorganisms (Schloss and Handelsman 2003). It is a science involving the application of shotgun sequencing directly to the sample obtained from our natural environment. It defies from the traditional genomics approach as it does not require

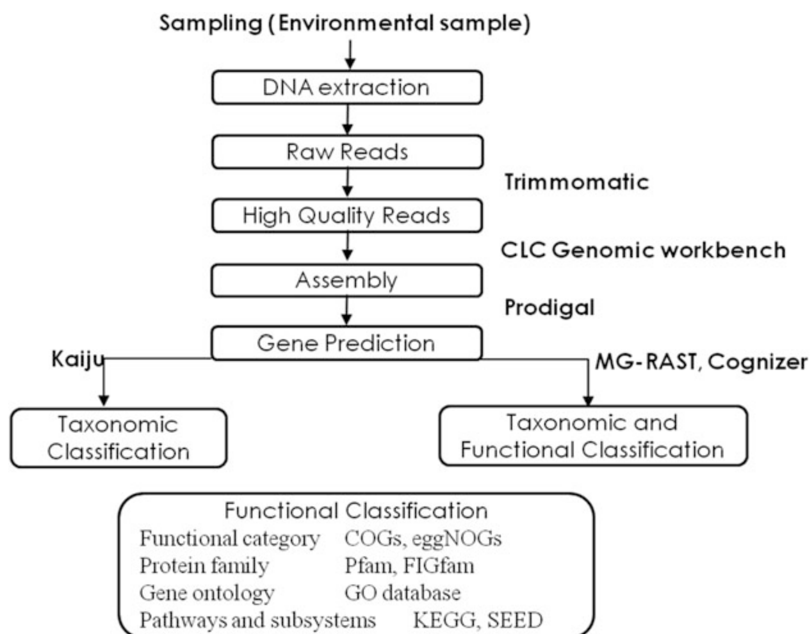

Fig. 14.2 The pipeline followed while performing metagenomics sequencing of a sample (both taxonomic and functional assessment)

pure microbial isolates for sequencing studies. Unlike the pure microbial genomics, metagenomics studies the microbial community as a whole, so this gives the possibility to also analyze the possible and fruitful interactions between the communities. With advancing technologies, the cost of sequencing these large genomes is also reaching within our limits. This will help us to know the unknown.

For resolution of the selected genome, a flexible pipeline is followed during the process. As shown in Fig. 14.2, it commences with sample collection (metadata is also assembled), from which the whole community DNA is extracted. After this, it leads to formation of library, sequencing, read preprocessing, and assembly (Kunin et al. 2008). Genes are then referred to as either reads or contigs, and binning is performed. A number of reference databases are used to ease the analysis. Different parameters can influence the metagenomics study due to which there are certain considerations which require attention.

(a) **Considerations Before Sequencing**

Every new science comes with its limitations. The feasibility of the metagenomics study is very much narrowed by its expensive operational cost due to the large genome sizes and low gene densities of the involved microbes (Thomas 1971). The genomic community involves bacteria, archaea, microbial eukaryotes, and viruses. Many a times, varying ecological spaces comprise of all the four groups

together at a single space, while sequencing all of them is cost-prohibitive for us. The major hindrance is due to the involvement of protists. While performing sequencing, it should be a priority to avoid a sample comprising eukaryotes keeping in consideration our existing sequencing capabilities. But this will exclude us to study the community entirely. Possible alternative is to narrow down the sequencing to RNA (metatranscriptomics) or protein level (metaproteomics) which will bypass the noncoding eukaryotic data (Kunin et al. 2008). The complexity of a community also defines the sequencing efficiency; less complex community will generate large contigs (continuous genomic stretches with overlapping reads) which are easy to annotate (Kunin et al. 2008).

(b) **Sequencing Technology and How Much to Be Sequenced?**

There are many new technologies existing in the scientific market (e.g., shotgun sequencing, pyrosequencing), and many more are under trial to deal with the hefty sequencing costs (Margulies et al. 2005). Each innovation extends to its advantages and disadvantages, so a combination of sequencing technologies would prove useful (like Illumina http://www.illumina.com). This would help us to achieve good sequencing quality and quantity.

Metagenome is endless. The data required to be sequenced should be decided following a rational approach and available budget. The essential genome coverage depends upon whether we want to analyze under- or over-represented genes. The specific genes involving the single-nucleotide polymorphism (SNP) will require a larger coverage of the genome in comparison with the overexpressed genes present in the community as a whole. The following section details a general process of metagenomics sequencing which passes through a well-defined pipeline.

(i) **Sample Collection**

Sample collection for whole DNA extraction should be efficient enough not to compromise with the succeeding sequencing. Low yields of DNA can hamper the library construction, for which amplification may provide a solution (Angly et al. 2006). Metadata compilation for the site like the biochemical characteristics, e.g., temperature, pH, geospatial coordinates, etc., facilitates to decipher the sequencing well.

(ii) **Shotgun Library Preparation, Sequencing, and Processing**

After the extraction of pure community DNA, it is used further to prepare library using DNA clones of different sizes (3, 8, and 40 kbp fosmids). A certain concentration of DNA is required (5–20 μg) to efficiently achieve this step. Following this step, DNA sequencing is carried out. During the analysis, two-stage quality control is performed, preliminary [only assembly and no gene prediction] and final [involving both] (Kunin et al. 2008).

Processing the Sequence Broadly Consists of Three Major Steps

(a) **Pre-processing**

Metagenomes which more often comprise of complex communities are more prone to error owing to their less coverage in sequential study. In the preprocessing step, base calling (recognizing DNA bases), removal of cloning vectors, sequence contamination, and low-quality bases (Trimming) are performed (Kunin et al. 2008). This makes it a very important step to avoid many possible downstream consequences which otherwise are often overlooked.

(b) **Assembly and Finishing**

This step involves the formation of contiguous stretches of DNA sequence using the similar reads termed as Contigs. The highest quality or most frequently occurring nucleotide serves the basis while preparing contigs. They are further assembled to from larger noncontiguous DNA sequence called scaffolds. Various computer software programs are available to assemble the reads like Arachne (Batzoglou et al. 2002; Jaffe et al. 2003), Phrap (www.phrap.org), the Celera Assembler (Myers et al. 2000), etc. All of these assemblers are prone to make mistakes, so we should always rely on a final manual inspection using visualization tools like Consed (Gordon et al. 1998), etc.

Closure of a genome is called as finishing which is really uncommon with metagenomes. This is only possible if we select a sample with low spatial heterogeneity or perform a specific and narrow-scale sampling to dodge it (Hallam et al. 2006).

(c) **Gene Prediction (or Gene Calling) and Annotation**

Post assembly, various generated contigs would be further identified for their protein and RNA sequence coded on the DNA which is termed as gene prediction. This step can also process the reads of the unassembled metagenome. There are basically **two approaches** used while predicting the genes.

(i) *Evidence-Based Gene Prediction* – In this, homology search is employed to predict the gene by performing BLAST comparisons against already available protein databanks. This has few limitations like due to large evolutionary distance, sequence errors, short length coding sequence, etc., many novel genes are completely ignored.

(ii) *"Ab Initio" Gene Calling* – This means "starting from the beginning." Nonhomologous genes are identified using the intrinsic capability of the DNA to differentiate between coding and noncoding regions. This approach usually generates large interfering amount of data with too much noise.

Researchers preferably employ a combination of both the abovementioned approaches like in the pipeline mORFind. Post-gene prediction, functional annotation has to be achieved. For this, using RPS-BLAST (Markowitz et al. 2006), protein sequences are compared to sequence alignment profiles from the protein families PFAM (Finn et al. 2008), TIGRFAM (Selengut et al. 2007), and

COGs (Tatusov et al. 1997; Kunin et al. 2008). Other methods for functional context annotations involve gene fusion (Enright et al. 1999; Marcotte et al. 1999a, b), coexpression (Marcotte et al. 1999a, b), etc.

(iii) **Data Analysis**

1. **Community Composition and Binning**

Informative and phylogenetically preserved marker genes like 16S and 23S rRNAs and RecA (DNA repair protein) are mapped to the respective reference trees to examine the organism identity and its abundance (Venter et al. 2004). The process proceeds from preparing a gene alignment using a reference dataset which is further identified owing to its specific marker genes. The community composition is not completely certain due to reasons like incomplete reference dataset, fragmented assemblies, erroneous gene prediction, etc. (Kunin et al. 2008).With advancing technologies, this field is under trial to develop more reliable and reproducible software like MEGAN (Huson et al. 2007).

Binning is a process of establishing an association between sequence data and different taxonomic groups (Kunin et al. 2008). Community composition and binning share a common goal of classification and even faces the same limitations. This classification further eases the profiling process to identify the major dominant populations which majorly governs the metagenome.

2. **Taxonomic and Functional Assessment**

Once the sequence alignment for the composite nucleotide sample is performed, it is followed by data analysis using a reference database. The sequences are annotated both taxonomically and functionally. With the help of software like Kaiju (Menzel et al. 2016) and MG-RAST (Metagenomic Rapid Annotations using Subsystems Technology; Keegan et al. 2016), sensitive taxonomic classification for the reads obtained from high-throughput sequencing is performed (Fig. 14.3). Different microorganisms belonging to the five kingdom classification are sorted out of the sample, and their percentage abundance is tabulated. Similarly, for the functional annotation, using a reference dataset, genes are classified to categories like metabolism, cellular processes, genetic and environmental information processing, human diseases, drug development, etc. (Fig. 14.4). These are sub-categorized to narrower classifications like carbohydrate metabolism, translation, cell growth, immune system, ion channels, etc. using software like KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (clusters of orthologs genes), MG-RAST, etc. Using the sequencing studies, the genes of interest, for example, those which are involved in degradation of aromatic toxic compounds, are selected for investigation and further employed in remediation of waste sites (Gupta et al. 2017).

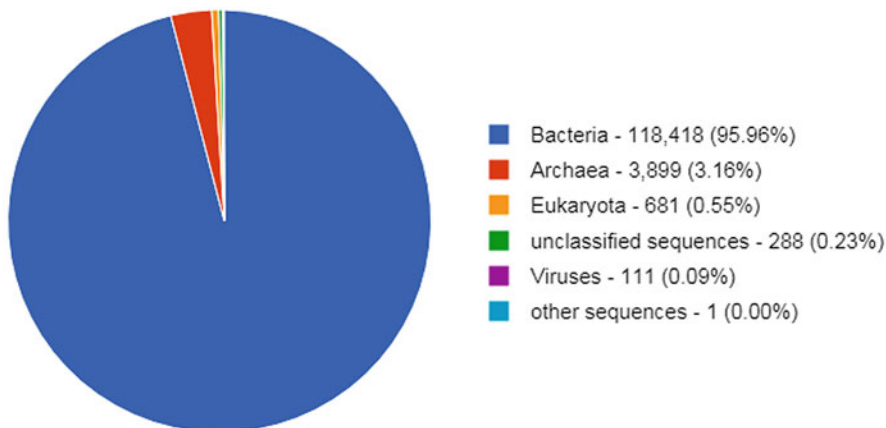

Fig. 14.3 Taxonomic profile of a waste site, revealing the prominent domain with their number of sequences and percentage abundance

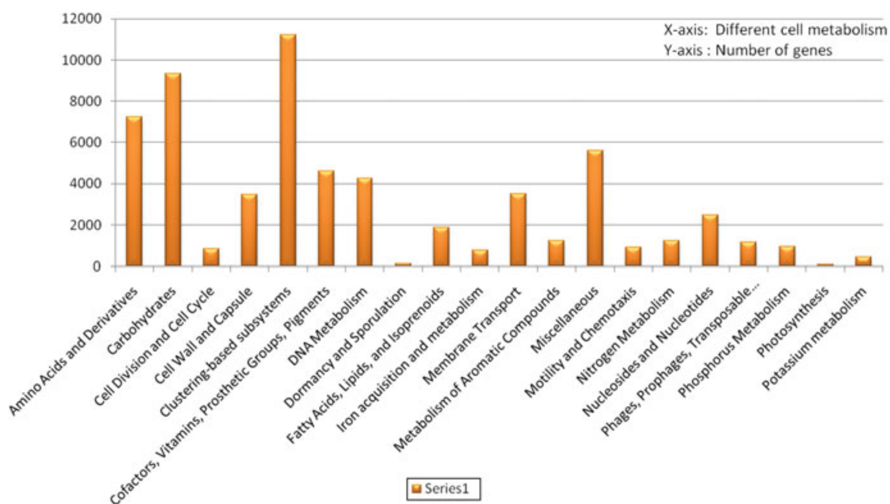

Fig. 14.4 Functional annotation of a microbial community deciphered from a waste site against a reference database. Genes involved in various metabolic processes are plotted

3. **Dominant Population and Functional Gene Abundance at Waste Sites**

Owing to their significant abundance, the three major groups *Actinobacteria*, *Proteobacteria* (*Betaproteobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria*), and *Firmicutes* are found at all sites. Similarly, the diversity at these waste sites is also dominated by a consortium of gram-positive and gram-negative strains (Stamps

et al. 2016). Numerous lineages of *Syntrophobacterales*, *Desulfovibrionales*, *Desulfuromonadales*, *Campylobacterales*, *Burkholderiales*, *Hydrogenophilales*, *Pseudomonadales*, *Clostridiales*, *Bacteroidales*, etc. are also observed. Archaeal members are found in relatively lower percentages, with *Euryarchaeota* being the most prominent one. There is a significant percentage of reads classified to the virus domain, and some are unclassified. Literature demonstrates that low quality reads, short assembly, inefficient sequencing, large genome size etc. are responsible for generating unclassified reads. At waste sites, dominance of metabolism genes, transporter genes, degradation enzymes, and stress-related functioning genes is also found.

The new sequencing technologies will help us unravel various potential microbial strains which can be used in bioremediation of environmental samples, for example, wastewater decontamination, heavy metal removal, etc. (Medhi et al. 2018), and even promote a stable ecosystem (Mishra et al. 2018a, b). The gene of interest can be molecularly cloned and transformed to a new fast-growing microbe. This will increase the metabolic efficiency of the selected gene and ultimately will help us to realize the potential of microbes in waste management using the culture-independent technology.

14.6 Present and Future Scenario of Waste Management

India is facing important environmental challenges including waste generation and inappropriate collection of waste, treatment, transport, and disposal. The volume of waste generated by an urban population is increasing which would pose a severe impact on the ecosystem and public health. By the year 2025, waste generation is expected to rise approximately to 27 billion tonnes, one-third contributor of which is Asia (Modak et al. 2010).

The current waste management process lags behind in many innovations and alternatives. We deal with this upsurging waste using physical, chemical, and biological methods. Different segregated bin collection systems, usage of optical sensors, and involvement of GIS technologies have been proposed and used. While treating the waste biologically, we use culture-dependent as well as culture-independent approaches of microbiology. Both the taxonomic and functional potential of microbes is explored and utilized.

Waste can be considered as a resource which might be used to extract energy. Proper management of waste can be achieved by recycling, reusing, and recovery of important substances from landfill mining (ISWA 2012). India needs proper rule and their implementation to resolve the problem associated with waste management and landfills. For energy generation from landfills, thermal treatment of waste is one of the many important opportunities, but it requires qualified engineers having experience in this field. Investments over such qualified minds are still lacking in India.

14.6.1 Waste Management Barriers and Opportunities in India

14.6.1.1 Waste Management Legislations

MSW (municipal solid waste) Handling and Management Rules 2000, issued by MoEF (Ministry of Environment and Forests), are responsible for dealing with the rising waste and should be given political priority. Various governmental bodies like MCD (Municipal Corporation of Delhi), CPCB (Central Pollution Control Board), etc., keep a check over the regulation of such acts and rules to ensure their proper implementation.

A strong and independent body with an efficient policy framework is required to work over the waste regulations in a directed way. Cost penalties should also be imposed on failing the management services; this would generate the required waste tax to be used later.

14.6.1.2 Waste Collection, Transport, and Disposal (3Rs)

The 3Rs are major steps for waste management. Proper and segregated collection would help in an efficient transport and disposal (Sharholy et al. 2007). Landfills and waste burning are common dumping options, but before dumping, the 3Rs should be always considered, reduce, reuse, and recycle to the maximum, which will help us eliminate the further issues of waste disposal.

We should treat waste as a resource, working over its extraction and recovery significantly. Over the last few years, many states of South India showed a remarkable increase in waste production, but following some simple steps like 3Rs and door-to-door collection presented the best example of waste dealing practices as well. Their slogan Nirmala Bhavanam, Nirmala Nagaram (Clean Home, Clean City) worked efficiently.

14.6.1.3 Concept of Engineered Landfills

Landfills are preferably the most common dumping option, but an un-engineered landfill site poses a number of threats. These include harmful air emissions, air-blown litter, leachate pollution to groundwater and surface water, odor issues, heat shock dominance, etc. (UNEP [United Nations Environment Programme] 2005; Ghosh et al. 2014). Various health-related problems including respiratory difficulties, allergies, and asthma also result due to poor management (CPCB 2000a, b, c). A properly managed landfill site is required for the complete well-being of humans, plants, animals, as well as the ecosystem.

14.6.1.4 Role of Informal Sectors

The role of informal sectors is very significant and should be clubbed with formal sector as well (Mathur 2012). Rag pickers play a noteworthy role in waste management at an informal level, and this waste is responsible for maintaining a stable livelihood for them. According to data inventory, every tonne of material recycled will save approximately 25,000 INR per year, avoiding the release of around 700 Kg CO_2 in the atmosphere (Annepu 2012).

14.6.1.5 Involvement of Public Sectors

Currently, private sector also shows great involvement in waste management. Cities like Mysuru present a well-defined example, using the door-to-door waste collection system. Apart from this, we have different research organizations and research-based NGOs which are dedicated to waste management like Green Peace, India.

14.6.1.6 Training and Capacity Building at Each Level

With the aid of various governmental and nongovernmental organizations, each individual especially students should be trained to realize the importance of waste management. Seminars, workshops, etc. should be regularly conducted in order to create awareness among the young minds.

14.6.1.7 Waste to Energy

As discussed earlier, like all the major developed nations, we should also consider our waste as a resource for our ecosystem. Mitigating the waste problems would become more favorable if we will shift our focus to material recovery from waste like the production of biodiesel, extracellular polymeric substances, etc. (Gupta and Thakur 2016; Khosla et al. 2017). Production of energy from waste would help in utilizing the free land for better alternatives other than usual dumping (Planning Commission, Government of India 2014). Energy recovery depends mainly on waste composition, while climatic, demographic, and socioeconomic factors also play their role (Gómez et al. 2009; Srivastava et al. 2014). Integrating maximum recycling with waste to energy technologies would provide us the main benefit. Numerous projects of generating energy from waste involving combustion of unsegregated low-calorific waste are under development, and different thermal processes like gasification, production of refuse-derived fuel, and gas-plasma technology are also considered. These wastes to energy options would also bring socioeconomic advantages to India. On the contrary, there are many limitations faced while generating energy from waste due to poor segregation, failed operations, and infrastructure, etc., but still, it will prove to be a keystone of future waste management (Indo-UK Seminar Report 2015).

A long-term future plan analyzes the present situation clubbing it with the upcoming conditions and challenges. For waste management, there are various parameters which demand our consideration like source, sink, waste characteristics, generation rate, and the possible combating alternatives (Kaushal et al. 2012). Many researchers are trying to address the issue, but due to the increasing trends of waste generation, it is quite challenging. A designed proposal would be more realistic if it gives us both pessimistic and optimistic values. To combat the overflowing waste, a life cycle assessment (LCA) of waste sites is a must. LCA will evaluate a waste site starting from its cradle to its grave. Various models like least-squares regression, saturation curve method, etc. discuss about the cause-effect relationships to further verify the innate systemic characteristics. According to the projected and available data for waste generation in Delhi between the years 1971 and 2024, it was concluded that inert and compostable wastes show decreasing trends, while paper, plastic, and glass materials show increasing trends (Kumar JS 2011). Similarly,

using such inventories, a statistical analysis for the parameter of interest is done, and changes are incorporated further. The prediction trends for the future are also devised using the present waste statistics.

14.7 Conclusions

The chapter discusses the significant issue of increasing solid waste influenced by a number of parameters: population explosion, rapid industrialization, and urbanization. The amount of waste would soon cross the limits and will rise up higher against various other global problems. An immediate need of the hour is to develop an organized and efficient solid waste management system, minimizing the accumulation and improving the solid waste conversion to energy. Different physical and chemicals methods are used for management, while the biological treatment of waste is still underestimated. Microbes are capable of degrading a wide range of compounds because of their physiological ability and metabolic efficiency. Microbiology employs both cultural-dependent and culture-independent approaches during bioremediation. Using cultural techniques, waste-contaminated sites with gasoline, diesel, oil, organic pollutants, chlorinated and nitrated compounds, pesticides, lignocellulosic waste, etc. are successfully bioremediated using different methods of in situ and ex situ remediation. While the culture-independent approaches use sequencing studies to identify the functional and taxonomic aspects of a site against a reference database. This will help us to realize the microbial potential of the hidden domains which can be further exploited according to the area of interest. Waste management faces many challenges in terms of lack of planning, improper regulations, involvement of stakeholders, etc. Innovation in waste management is limited by the lack of motivation and environmental awareness. Environmentalists should inform the common public about the menace that the waste can create. A positive change in public attitude can bring colossal prospects.

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Diversity of Polysaccharides in Cyanobacteria

15

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Abstract

Polysaccharides show immense structural variability by virtue of their monomer composition, linkages, oligomer units, branching, size, and interactions with non-saccharide components. In cyanobacteria, polysaccharides are found as storage molecules, in cell envelopes, and as extracellular polysaccharides (EPS). Storage molecules exist as glycogen and cyanobacterial starch and exhibit lowest diversity. As part of the cell envelope, lipopolysaccharides (LPS) in the outer membrane contribute 70–75% to the cyanobacterial cell surface. O-antigen polysaccharide imparts structural heterogeneity and thus strains specificity even in the cyanobacterial species sharing the same habitat. LPS is responsible for a diverse range of health effects in man. EPS that interfaces with the surrounding environment shows maximal structural diversity and functional versatility. Functions of the EPS vary with the species and provide as the primary mechanism for survival in extremes, defence against toxins, heavy metals, predators, and other antagonists. They modify fluidity of the external milieu and are involved in cellular communication important in structuring the biofilm community. In fact, both survival and growth of the organism are dependent on the organisms' EPS arsenal. Thus, the cyanobacteria spend up to 70% of the total energy reserve in the production of EPS. Such diversity of polysaccharides is not easy to be replicated through synthetic processes. This chapter provides glimpses of the diversity of polysaccharides found in cyanobacteria and their industrial potential to encourage prospective work in this area.

Keywords

Cyanobacteria · Extracellular polysaccharides · Glycogen · Lipopolysaccharides · Starch · Semiamylopectin

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15.1 Introduction

Cyanobacteria are efficient at solar energy capture investing as much as 9% of the solar energy into biomass as compared to only 0.5–3% for higher plants (Dismukes et al. 2008; Branco dos Santos et al. 2014). They can fix an estimated 25 gigatons of carbon from CO_2 per year into energy dense biomass (Paumann et al. 2005) constituting 0.05% of global carbon in biomass (Garcia-Pichel et al. 2003). Cyanobacteria have existed since the Proterozoic era (2500–570 Ma) where they were the principal primary producers and the ultimate source of atmospheric oxygen (Schopf and Walter 1982). The transition from a reductive to an oxidative environment triggered diversification of cyanobacterial lineages and appearance of new traits (Schirrmeister et al. 2016). The group has acquired remarkable adaptations in the evolutionary journey establishing them in the most diverse aquatic and terrestrial environments across the latitudes, from the polar to the tropical, along all altitudes and extremes and in a variety of ecological associations. Polysaccharides have played a critical role in establishing these communities in the process.

Diverse structures can be created by simply linking different monosaccharides through glycosidic bonds, different conformations, configurations, branching, and interactions with other non-saccharidic components that further generate macromolecular, structural, and functional versatility to the roles that they perform. Polysaccharides by nature are designed to perform various specific functions in a living organism. They usually act as carbon sinks that provide energy reserve; maintain structural integrity; alleviate stress; defend against toxins, parasites, and preys; and act as information systems (Lohman 1990). Minor modifications in the structure can cause major changes in the properties and attributes of the polysaccharide. Remarkably, these modifications may be brought about in response to as little changes in the abiotic and biotic factors. Cyanobacteria produce polysaccharides either endogenously serving as storage polysaccharides as part of the cell wall or exogenously, and discussion on these components is the primary focus of this review.

15.2 Cell Wall Polysaccharides

Cyanobacterial cell walls resemble the Gram-negative bacterial architecture comprising of cytoplasmic membrane, peptidoglycan layer, and an outer membrane. Though the overall structure of cyanobacterial cell wall is that of a Gram-negative wall, the peptidoglycan layer is considerably thicker resembling a Gram-positive wall. In unicellular strains like *Synechococcus* sp., the layer is about 10 nm thick, and the filamentous forms like *Phormidium uncinatum* have a 15–35-nm-thick peptidoglycan, while larger forms like *Oscillatoria princeps* have a 700-nm-thick layer. The extent of crosslinking is also high: 55–63% in cyanobacteria as against 20–33% in Gram-negative bacteria (reviewed by Hoiczyk and Hansel 2000). The outer membrane is composed of lipopolysaccharides that are amphiphilic heteropolymers comprising 10–15% of the outer membrane and covering nearly

75% of the total cell surface (Lerouge and Vanderleyden 2002). They are heat-stable endotoxins and have been recognized as a key factor in septic shock in humans. LPS contributes to the structural properties of the cell envelope and acts as a physical barrier to protect the cell. More external layers like the capsule, S layer, sheath, and slime that occur above the outer membrane along with the cell wall are annotated as the cell envelope. Additionally, cell type-specific structures also exist like glycolipid layer and polysaccharide layer around the heterocysts of filamentous cyanobacteria (Herrero et al. 2016).

15.3 Lipopolysaccharides

Lipopolysaccharides (LPS) are highly acylated saccharolipids with a molecular mass of about 10–20 kDa (Lerouge and Vanderleyden 2002). This complex amphiphilic macromolecule is composed of a glucosamine disaccharide backbone with hexa- or hepta-acyl chains, linked to a hydrophilic polysaccharidic core that extends out to the environment (Raetz and Whitfield 2002). The O-antigen consists of repetitive subunits that extend out from the bacteria and can include from 1 to 25 chemically identical repeating oligosaccharide units, which, in turn, contain 2 to 7 monosaccharide residues, generally hexoses (Wilkinson 1996). The polysaccharide chains show heterogeneity in terms of monosaccharide composition, their alternative configurations, and the innumerable types of glycosidic linkage, length, branching degrees, and noncarbohydrate substituents. It exhibits interstrain as well as intrastrain heterogeneity and is the basis of serological and antigenic specificity of the organism (Lerouge and Vanderleyden 2002). Presence of O-antigen modifies the appearance of a bacterial colony from rough to smooth. Another morphological variant is called “semi-rough” and contains short-chain-type LPS having only one O-chain repeating unit (Nazarenko et al. 2011).

15.3.1 Core

The core region is less varied than that of O-antigens comprising up to 15 sugar residues and responsible for antigenicity in the rough-type LPS (Caroff and Karibian 2003; Steimle et al. 2016). It is divided into a proximal and a distal region. The proximal region, called “inner core,” contains 3-deoxy D-manno-oct-2-ulosonic acid (Kdo), heptoses, and negative charges usually derived from phosphate groups, and it is important for maintaining the integrity of the outer membrane. The distal region, called “outer core,” provides attachment to the O-antigen, if present, and is usually composed of hexoses and shows more structural variability (Caroff et al. 2002; Caroff and Karibian 2003; Gemma et al. 2016). The core region is further linked to lipid A via a Kdo residue. Usually, the core region contains L-glycero-D-manno-heptose (L,D-Hep) and an L-α-D-Hep(1,3)L-α-D-Hep(1,5) [α Kdo (2,4)] α-Kdo tetrasaccharide (Hep II, Hep I, Kdo II, and Kdo I, respectively), which may be further substituted by other sugars or phosphate residues or sometimes by acetyl

groups or amino acids. In addition to L, D-Hep, several LPS contain its biosynthetic precursor, D-glycerol-D-manno-heptose (D, D-Hep). There are other LPS that contain only D, D-Hep or even lack any heptose. Kdo may be replaced by the stereochemically similar sugar acid D-glycero-D-talo-oct-2-ulosonic acid (Ko) (reviewed by Holst 2011).

15.3.2 Backbone

Lipid A (endotoxin), a glycophospholipid that provides anchorage to the molecule in the outer membrane, is composed of a glucosamine disaccharide backbone in 1,6 linkage and is a highly conserved segment. At 1′ and 4′ positions, the disaccharide contains α-glycosidic and nonglycosidic anionic phosphoryl groups, and at positions O 2, O 3, O 2′, and O 3′ are (R) 3-hydroxy fatty acids in ester and amide linkages. Two of these fatty acids are usually further acylated at their 3-hydroxyl group. Most of the bacteria show acylation with 4–6 chains ranging from 10 to 16 carbon atoms in length. The type of hexosamine present, degree of phosphorylation, the presence of phosphate substituents, chain length, number, and position of the acyl groups impart individuality to the cells (Kabanov and Prokheronko 2010; Steimle et al. 2016).

15.3.3 Cyanobacterial Lipopolysaccharides

Cyanobacterial lipopolysaccharides are structurally and functionally different from the proteobacteria (Hoiczyk and Hansel 2000; Snyder et al. 2009). Most cyanobacteria possess a simplified LPS structure containing 31–80% carbohydrates, 8–18% fatty acids, and 0.1–8% proteins (Durai et al. 2015). There are none or trace amounts of 3-deoxy-D-manno-oct-2-ulosonic acid (KDO), which is ubiquitously present in enteric Gram-negative bacteria. But some strains of cyanobacteria, viz., *Spirulina platensis* (Tornabene et al. 1985), *Microcystis aeruginosa* NRC1 (Raziuddin et al. 1983), *Phormidium* sp. (Mikheyskaya et al. 1977), *Anacystis nidulans* (*Synechococcus* PCC 6301) (Katz et al. 1977), and *Agmenellum quadruplicatum* (*Synechococcus* PCC73109) (Buttke and Ingram 1975), possess KDO.

Heptoses are absent in most cyanobacteria. Some cyanobacteria do not have phosphates, while others show its presence in variable amounts (Weckesser et al. 1974, 1979; Schmidt et al. 1980a, b; Keleti and Sykora 1982; Carillo et al. 2014; Simkovsky et al. 2016). Unlike Gram-negative LPS, the presence of galactose and glucosamine is also variable. Studies have indicated that neutral sugars like rhamnose, fucose, xylose, mannose, galactose, and glucose are conserved among most of the cyanobacterial species. Immense variability exists down to the chemotype (Schmidt et al. 1980a, b). The LPS molecules also contain relatively large quantities of oleic, palmitoleic, linoleic, and linolenic acids that are typically absent in Gram-negative LPS molecules. They lack phosphate residues and instead have a single galacturonic acid attached to glucosamine.

The carbohydrate region in *Anacystis nidulans* is comprised of fucose, galactose, glucose, mannose, rhamnose, KDO (2-keto-3-deoxy-octonic acid), glucosamine, and 2-amino-2-deoxy-heptose (Weise et al. 1970). Katz et al. (1977) reported the presence of KDO and β-hydroxymyristic acid in *A. nidulans* (*Synechococcus* PCC 6301), which are also seen in LPS of Gram-negative bacteria. However, it lacked heptose and had phosphate and glucosamine in small amounts in its lipid moiety. Besides the common core sugars and xylose, there is L-acofriose in *Anabaena variabilis*, fucose in *Anabaena flos-aquae*, and 3,6-dideoxyhexose in *Anabaena cylindrica* (Keleti and Sykora 1982), and galacturonic acid as the main component in the core oligosaccharide of *Oscillatoria planktothrix* FP1 (Durai et al. 2015). LPS in *Schizothrix calcicola* contained neutral sugars, viz., galactose, glucose, mannose, rhamnose, xylose, and glucosamine as the only amino sugar without any KDO and heptose (Keleti et al. 1979).

Snyder et al. (2009) working on *Synechococcus* sp. observed that the core region was primarily composed of a 1,4-linked glucose chain with low levels of glucosamine and galacturonic acid. Its strain WH8102 also had a single rhamnose. Raziuddin et al. (1983) reported substantial amounts of KDO, glucose and other hexoses, 3-deoxy sugars, glucosamine, fatty acids and their esters, and phosphates in the LPS of *Microcystis aeruginosa* NRC1, while Martin et al. (1989) reported absence of KDO and heptoses in two strains of *M. aeruginosa*, PCC 7806 and UV-017. A study by Fujii et al. (2012) on the O-chain of *Microcystis aeruginosa* reported glucose (66%), rhamnose, xylose, mannose, and galactose and that of *M. aeruginosa* NIES-87 was found to be composed of glucose alone. It suggested that glucose (and its derivative) being the sole monosaccharide component in the O-chain of *M. aeruginosa* may imply that the functional roles of the O-chain might differ from its role in proteobacteria.

The O-antigen of *Synechococcus elongatus* PCC 6301 is reminiscent of the polymannose O-antigen of *Escherichia coli* O8 and O9 (Katz et al. 1977). LPS of *Agmenellum quadruplicatum* was found to be unique due to the presence of xylose in the polar heads and unusual pentoses in the O-antigen, while galactose was absent. Presence of rhamnose and mannose along with absence of heptoses conformed with common cyanobacterial LPS structures (Durai et al. 2015). Sugar analysis of the LPS of *Spirulina platensis* showed presence of common core sugars as glucose, KDO, rhamnose, mannose, galactose, fucose, ribose, and xylose, along with a variety of unique sugars such as inositol, D-glycero-D-manno-heptose, D-glycero-L-mannoheptose, and 3- or 4-O methylhexoses and glucosamine as the lone amino sugar. Minor quantities of 3-hydroxy palmitic acid were also detected (Tornabene et al. 1985). Sugar composition of some of the cyanobacterial polysaccharides is presented in Table 15.1.

Lipid A is an acylated glycolipid that anchors the LPS molecule in the outer membrane of the Gram-negative bacteria and is the most conserved biochemical structure of this group of organisms (Stewart et al. 2006). Its fatty acid composition is reported to be highly heterogeneous both in terms of length and degree of saturation, ranging from lauric acid (C12) to stearic acid (C18) along with other polyunsaturated fatty acids like linoleic and linolenic acid. Such long- chain fatty

Table 15.1 Sugar composition of cyanobacterial lipopolysaccharides

Organism	Glu	Gal	Man	Xyl	Rha	Fuc	GluA	maA	KDO	Others	References
Anabaena cylindrica	+	+	+	+	+		+			3,6-Dideoxyhexose	Keleti and Sykora (1982)
Oscillatoria brevis	+	+	+	+	+						
O. tenuis	+		+	+	+				L	Heptose present	
Anabaena variabilis (*Anabaena* PCC7118)	+	+	+		+		+			Acofriose, phosphate present	
Anabaena flosaquae UTEX 1444	+	+	+	+	+	+	+		L		
Schizothrix calcicola	+	+	+	+	+		+				Keleti et al. (1979)
Spirulina platensis	+	+	+	+	+	+	+		+	Inositol, ribose, D-glycerol D-mannoheptose, glyceromannoheptose, 3- or 4-O-methylhexose	Tornabene et al. (1985)
Synechococcus sp. strains C9311	+		+	+	L		+			Galacturonic acid	Snyder et al. (2009)
WH8102	+		L		+					Galacturonic acid	Schmidt et al. (1980a, b)
6907	+	+	+		+	+	+				
6307	+	+	+		+	+	+				
6911	+	+	+		+	+	+				
6603	+	+	+			+	+				
6908	+	+	+			+	+				
6311	+	+	+			+	+				
6312	+	+	+			+	+				
6910	+	+	+			+	+				
Synechocystis PCC 6714	+	+	+			+	+				
6803	+	+	+			+	+				

6807	+	+	+		+	+	+				
6308	+	+	+		+	+	+				
Anacystis nidulans (*Synechococcus* PCC 6301)	+	+	+			+	+	+	+	Methyl-D-mannose	
Microcystis aeruginosa NRC1	+						+		+	3-Deoxy sugar, glyceromannoheptose, phosphate present	Raziuddin et al. (1983)
Microcystis aeruginosa NIES 87	+	+	+	+	+						Fujii et al. (2012)
M. aeruginosa PCC 7806	+	+	+	+	+	+	+				Martin et al. (1989)
PCC 7820	+	+	+	+	+	+	+				
UV 017	+	+	+	+	+	+	+				
UV006	+						+				
(*Synechococcus* PCC73109) *Agmanellum quadruplicatum*	+	+	+	+	+		+		+		Buttke and Ingram (1975)
Anacystis nidulans KM	+	+	+			+	+	+	+		Katz et al. (1977)
Phormidium	+	+	+	+	+		+		+	Galactosamine, arabinose, acofriose, phosphate present	Mikheskaya et al. (1977)
Oscillatoria planktothrix			+		+		+			Galacturonic acid	Carillo et al. (2014)

Glu glucose, *Gal* galactose, *Man* mannose, *Xyl* xylose, *Rha* rhamnose, *Fuc* fucose, *GluA* glucosamine, *GalA* galactosamine, *maA* mannosamine, *KDO* 3-deoxy-D-manno-octulosonic acid

acids and polyunsaturated fatty acids are mostly not known in the LPS of Gram-negative bacteria (Weise et al. 1970; Buttke and Ingram 1975; Keleti et al. 1979; Keleti and Sykora 1982; Tornabene et al. 1985; Martin et al. 1989). Only a few studies are covered here to let the reader form a picture of the entire LPS in cyanobacteria as lipid A requires a separate review.

Cyanobacterial lipopolysaccharides contain large amounts of oleic, palmitoleic, linoleic, and sometimes linolenic acids also (Keleti and Sykora 1982). Snyder et al. (2009) found that the lipid moieties in *Synechococcus* sp. had tri- and tetra-acylated structures with odd-chain hydroxy and nonhydroxy fatty acids connected to the diglucosamine backbone. In line with other cyanobacteria, LPS of *Oscillatoria planktothrix* FP1 also had no KDO, heptose and phosphate; however, hydroxylated and nonhydroxylated fatty acids have been reported in the glucosamine disaccharidic backbone (Carillo et al. 2014). Digalactosyl diacylglycerol and phosphatidyl diacylglycerol along with unsaturated fatty acids and 3-hydroxy myristate were observed by Tornabene et al. (1985) in *Spirulina platensis*. The lipid A portion of *Schizothrix calcicola* is composed of β-hydroxylauric, β-hydroxypalmitic, linoleic, myristic, oleic, palmitic, pentadecanoic, and stearic acids (Keleti et al. 1979). LPS of *Agmenellum quadruplicatum* and *Anacystis nidulans* contain behenic acid along with β-hydroxy fatty acids analogous to other Gram-negative bacteria (Buttke and Ingram 1975). The lipid portion of LPS from another strain of *Anacystis nidulans* was composed of a series of long fatty acyl chains including β-hydroxymyristic acid (Weise et al. 1970).

15.4 Storage Polysaccharides

15.4.1 Glycogen

Glycogen is a dynamic form of glucose storage that combines low osmotic activity and accessibility to hydrosoluble enzymes. Typically, 5–15% of carbon fixed by cyanobacteria is stored as glycogen, and under certain conditions, it can contribute to up to 70% of dry biomass (Depraetere et al. 2015; Song et al. 2016). It is a highly branched, homogeneous, amorphous, water-soluble polyglucan composed of 9–13, (1,4)-linked α-D-glucose residue interlinked via (1,6)-α-D-glucosidic linkages. It forms a rigid granular structure of about 10^7–10^8 Da (Ball et al. 2011; Damrow et al. 2016) and serves as the main carbon sink and energy storage molecule in cyanobacteria. Each α-1,4-linked chain supports on an average two branched chains reaching 8–10% that are randomly arranged but densely packed and get progressively more crowded toward the periphery (Welkie et al. 2016). The size of the particle increases to a maximum possible diameter of 42 nm (Shearer and Graham 2002) containing up to 55,000 glucose residues with over 36% resting in the outer particle chains (Meléndez et al. 1999). These are readily accessible to cell metabolism without the need for polysaccharide debranching. There is abundance

of short chains with a degree of polymerization (DP) ≤ 8 (32–75%), and < 1% consisted of long chains with a DP ≥ 37 as observed by Meléndez et al. (1999) in *Synechococcus elongatus* PCC 7942.

Despite sharing the same chemical linkages, starch and glycogen differ widely in physicochemical properties. Starch granules are semicrystalline and insoluble in cytosol. They are usually made up of two α-glucan polymers, namely amylopectin and amylose. The minor fraction, amylose, is composed of linear weakly branched glucan chains (less than 1% of α-1,6 branches), while amylopectin, which is the major component, has the same basic structure but has considerably shorter chains and a lot of α-(1,6) branches. This results in a very complex, three-dimensional structure (Hizukuri 1986; Bertoft et al. 2010; Laohaphatanaleart et al. 2010). In amylopectin, α-1,6 glucosidic linkages are densely localized along the glucan chains with 9–10-nm intervals forming unit clusters. Double-helical structures are formed when the degree of polymerization (DP) approaches 10–20 glucosyl units within the cluster (Kainuma and French 1972; Gidley and Bulpin 1987), which are further closely packed with a radial orientation in a starch granule. The number of branches increases with an increase in radius, and consequently, concentric lamellae of alternating amorphous and crystalline regions are formed. The branching rate is nearly half (5%) of that observed in glycogen. Average DP reaches 10^4–10^8 glucose units per molecule corresponding to a molecular mass of 10^6–10^8 g mol^{-1} (Hizukuri et al. 1983; Takeda et al. 1988).

According to Konopka (1984), the formation of polysaccharide is a function of the relationship between energy generation and growth. It is induced when the energy generated is more than that needed for growth. Thus, polysaccharide formation results from overflow metabolism. The biosynthetic pathway of bacterial glycogen is very similar to that of starch biosynthesis in plants, using ADP-glucose as a major substrate for starch biosynthesis with different elongation properties for glucose extensions (Manners 1991; Sivak and Preiss 1998). The enzymes of glycogen metabolism are conserved in all cyanobacteria (Beck et al. 2012). Glycogen is synthesized by the sequential action of three enzymes: ADP-glucose pyrophosphorylase (AGPase) that activates the glucose to form ADP-glucose which is then polymerized to the nonreducing end of an α-1,4-linked glucan chain by glycogen synthase (GS) and the branching enzyme (BE) that introduces symmetrically distributed α-(1,6) glucosidic linkages according to a binary branching principle via a hydrolytic cleavage reaction. The tandem cluster structure of amylopectin is considered to be synthesized by concerted reactions catalyzed by three classes of enzymes, i.e. starch synthase (SS), starch branching enzyme, and starch debranching enzyme, each of which is composed of multiple isozymes making a different contribution to the cluster structure (Nakamura 2002; Ball and Morell 2003). In contrast, it was accepted that glycogen can be synthesized by a single form of glycogen synthase and glycogen branching enzyme in animals and bacteria. However, two types of glycogen synthases (GSI and GSII) have been reported in *Synechocystis* sp. PCC6803 with different elongation capacities. While GSI preferentially extends chains progressively by adding more glucose units to the same

chain, thereby generating longer branch chains in the glycogen structure, GSII adds single glucose units distributively one at a time to many chains adding intermediate-length chains instead (Yoo et al. 2014). Breakdown of the glycogen granule occurs through the actions of two enzymes, a debranching enzyme (DBE; GlgX) and the glycogen phosphorylase (GPase and GlgP) [Reviewed by Shearer and Graham 2002; Welkie et al. 2016].

Glycogen metabolism is under the control of circadian oscillator in *Synechococcus elongatus* PCC7942 (Suzuki et al. 2007), a phenomenon originally considered to be restricted to eukaryotic organisms (Diamond et al. 2015). When cyanobacteria are grown in a 24-h light:dark (LD) cycle, cells perform photosynthesis and accumulate glycogen during the day which provides for cell integrity, function, and viability during the dark period via the oxidative pentose phosphate cycle (Osanai et al. 2007). LD transitions involve changes in cytoplasmic pH and redox state, as well as changes in the intracellular concentration of specific metabolites and metal ions. These factors mainly regulate the switch between assimilatory (photosynthetic) and catabolic pathways in the cyanobacterial cell (Smith 1982). In fact, enzymes in glycogen metabolism are sensitive to the cellular redox state, and LD transitions alone may trigger changes in the glycogen content (Díaz-Troya et al. 2014).

Glycogen metabolism enables efficient energy homeostasis (Cano et al. 2018) acting as buffers and cellular tools for the compensation of stressful energetic transitions, mainly to ameliorate and avoid futile cycles during the process of changing photosynthetic activity and metabolic switching, as has been observed in metabolic networks of *Synechocystis* sp. where glycogen provides for all the precursors for biomass formation, metabolites, and cofactors in the dark (Puszynska and O'Shea 2017). Pattanayak et al. (2014) showed that glycogen in *S. elongatus* oscillates in continuous light conditions and that this oscillation depends on a functional clock that segregates pathways for storage and degradation of carbon temporally. Besides its role in maintenance metabolism under dark, glycogen is also involved in creating homeostasis in periods of starvation, nutrient deficiency, and salt and oxidative stress where again metabolic switching takes place (Suzuki et al. 2010; Zilliges 2014). Glycogen metabolism has also been associated with symbiotic performance, colonization, and virulence in bacteria, but such a role has not been reported in cyanobacteria (Wilson et al. 2010).

Though *Synechocystis* sp. and other forms in the order Chroococcales do not form a resting cell under stress, like species of the orders Nostocales and Stigonematales, these cells also switch stringently from an active photosynthetic protein status to a dormant glycogen status (Kaprelyants et al. 1993). Glycogen is known to accumulate under nitrogen deficiency. In *Arthrospira platensis*, its content increases from 13.7 to 63.2%, while the protein content decreases from 42.7 to 15.4%. *Synechocystis* PCC 6803 is capable of mixotrophic growth on glucose and stores the excess carbon as glycogen increasing intracellularly from 1 to 19 mg g wet cell^{-1} in a nitrogen-deficient medium (Yoo et al. 2007), while nitrogen deprivation with high light intensity (200 μmol photons m^{-2} s^{-1}) further enhances its concentration to 41.35 mg g wet cell^{-1} (Monshupanee and Inchroesakdi 2014). Growth conditions

also affect the structure of glycogen as observed in *Synechocystis* sp. PCC6803 (Yoo et al. 2007). Glycogen production under nitrogen limitation (0.084 g $NaNO_3$ L^{-1}) with 5 mM glucose yielded glycogen with a DP of 10.4, which increased to 10.7 two days after the cultures were transferred from a medium containing normal N concentration (1.5 g L^{-1}) and glucose to a nitrogen-limited glucose-supplemented medium. Glycogen synthesis mutants in another study were found to lose their viability on agar plates containing glucose (Gründel et al. 2012).

Enhanced glycogen production in response to nitrogen limitation has also been reported in *Spirulina maxima*, *Synechococcus* sp. strain PCC 7002, *Synechocystis s*p. strain PCC 6803, *Arthrospira platensis*, *Arthrospira maxima*, *Anabaena variabilis*, and *Anacystis nidulans* (Lehmann and Wöber 1976; Earnst and Boger 1985; De Philippis et al. 1992; Aoyama et al. 1997; Aikawa et al. 2012; Guerra et al. 2013; Hasunuma et al. 2013; Xu et al. 2013). Under nitrogen deficiency, other than photosynthesis, carbon skeleton of glycogen is probably derived from the amino acids released from proteins by gluconeogenesis. Along with accumulation of glycogen, cells undergo bleaching with concomitant breakdown of phycobilisomes and chlorosis (Hasunuma et al. 2013). The cells maintain residual photosynthesis (0.1% of the initial activity) (Sauer et al. 2001) allowing them to preserve full viability for over 6 months (Klotz et al. 2016). Similar long-term survival time has been reported for *Synechocystis* sp. also (Gründel et al. 2012). Mutants of *Synechocystis* sp. incapable of glycogen synthesis cannot perform metabolic switching, and thus, there is absence of chlorotic response while cells spill energy in the form of pyruvate and 2-oxoglutarate with 30–60% loss of carbon. Viability of cells on prolonged nitrogen starvation is lost in absence of glycogen. In the stenohaline cyanobacterium *Synechocystis* sp. PCC6803, a shift in osmotic response is observed in absence of glycogen synthesis with 29 times increase in sucrose synthesis under salt stress as glucosylglycerol, its primary osmolyte, could not be synthesized (Miao et al. 2003).

15.4.2 Semi-Amylopectin/Cyanobacterial Starch

Though soluble glycogen is the primary storage molecule in cyanobacteria, certain unicellular diazotrophs such as *Cyanothece* sp. ATCC 51142 and *Cyanobacterium* sp. CLg1 (Reddy et al. 1993; Falcón et al. 2004), *Synechococcus* sp. BG043511 (Ikemoto and Mitsui 1994), *Cyanobacterium* sp. MBIC10216 (formerly *Synechocystis aquatilis* SI-2), and *Cyanobacterium* sp. NBRC 102756 (Nakamura et al. 2005) contain within their cells numerous carbohydrate storage granules of distinct polysaccharidic nature that resemble amylopectin and thus were called semi-amylopectin. Contrary to the glycogen and phytoglycogen (rice endosperm), the cyanobacterial semiamylopectins were found to be slightly smaller in size (Nakamura et al. 2005) (Table 15.2). Semiamylopectins are composed of 2–6% long chains with a degree of polymerization of ≥ 37. Glycogen of *Synechococcus elongatus* PCC 7942 is composed of only 0.4% long chains in contrast to the rice endosperm that contains 6.2% long chains. The very short chains with a degree of

Table 15.2 Differences in glycogen and starch

S. No.	Property	Glycogen	Starch	Cyanobacterial starch
1	Basic unit	Glucose	2-α-Glucan polymers amylopectin (75%–88%) Amylose (20–25%)	Semiamylopectin, some also contain 5% amylose
2	Crystallinity	Amorphous	Semicrystalline	Semicrystalline
3	Branching	α-1,4-Glucan with 8–10% α-1,6 branching	Amylopectin : α-1,4-glucan with 5% α 1,6 branching Amylose: α-1,4-glucan, linear	α-1,4-Glucan, α-1,6 branching at intervals of 9–10 nm
4	Structure	Random arrangement Dense packing Crowded toward periphery	Tandem cluster arrangement: Branches densely localized along the chain forms. Unit clusters arranged in double helix, oriented radially in concentric rings	Tandem cluster amylose may or may not be present
5	Degree of polymerization (DP)	Most abundant average $(DP)_n$: 6–8 Short chain DP $\leq$8: 32–75% Long chain DP $\geq$37: <1% with 2 branches/ chain in 12 tiers. Up to 55,000 residues	$(DP)_n$ amylose: 11–12 $(DP)_n$ amylopectin: 20–30 DP $\geq$37: 6–7% (rice endosperm) DP $\leq$8: 7–8% up to ~two million residual molecules	$(DP)_n$ semiamylopectin: 11–12 DP $\geq$37: 2–6% DP $\leq$8: 7.5–25%
6	Particle diameter (nm)	Max 42 nm (Shearer & Graham 2002)	0.5–100 μm	0.2–0.7, Spherical or discoid granules
7	Solubility	Soluble in cytosol	Insoluble	Insoluble
8	Synthesis	ADPase, glycogen synthase (GSI GSII), BE	Multiple isozymes of starch synthase, starch branching and starch debranching enzymes	Isozymes reported AGPase, GS/SS, BE
9	Molecular mass	10^7–10^8 Da	Amylopectin: 10^8–10^{10} Da Amylose: 10^6–10^8 Da	Similar to amylopectin
10	Branching enzyme gene copies	1 or 2	1	3

Reference: Welkie et al. (2013, 2016), Meléndez et al. (1998, 1999), Suzuki and Suzuki (2013), Suzuki et al. (2013), Yoo (2001)

polymerization ≤8 were in a range between 7.5% and 25%. The proportion of the long to short chains in different species is intermediate between cyanobacterial glycogen and rice endosperm (Nakamura et al. 2005; Shimonaga et al. 2008; Hirabaru et al. 2010; Suzuki et al. 2013). A relative proportion of as low as 2% long glucan chains with a DP of ≥37 is enough for the macromolecule to achieve a cluster-like structure. The insoluble semiamylopectins form 0.2 to 0.7 μm spherical or disk-shaped granules with a tandem cluster structure. While *Cyanobacterium* sp. MBIC10216 polyglucan did not show the presence of amylase (Nakamura et al. 2005), starch-like granules in *Cyanobacterium* sp. CLg1 were found to be composed of both an amylopectin-like high mass fraction and a smaller amylose fraction (linear or scarcely branched (Suzuki et al. 2013)). The chain length distribution of the high-mass polysaccharide complies with the definition given for semiamylopectin, as it contains fewer of those chains exceeding a DP of 40 (Nakamura et al. 2005). Because the granules also contain a significant amount of amylose (5%), this material has been called cyanobacterial starch (Cenci et al. 2013). The average DP of amylose ranges from 11 to 12 which has also been reported for semi-amylopectin formed in cyanobacteria.

Analysis of storage polysaccharides from *Cyanothece* sp. ATCC 51142, *Cyanobacterium* strain Clg1, and *Cyanobacterium* strain NBRC 102756 revealed that their storage granules have a molecular mass virtually indistinguishable from that of amylopectin (Suzuki et al. 2013, 2015). Moreover, the thermal properties, crystallinity, and branching structure are similar to those of amylopectin, and the semi-amylopectin material synthesized by these strains is organized in tandem cluster structures. Isoforms have been reported for enzymes involved in the synthesis of cyanobacterial starch. The *Cyanothece* sp. ATCC 51142 has two genes each encoding ADP-glucose pyrophosphorylase (AGPase) and glycogen synthetase (GS)/starch synthase (SS) and three genes for the branching enzyme (BE). The presence of two GS/SS genes is observed in various species of cyanobacteria (Suzuki et al. 2010). Two genes for AGPase are found only in a few strains of *Cyanothece* and *Acaryochloris marina* and may not occur commonly among unicellular diazotrophic cyanobacteria (Suzuki et al. 2013).

15.5 Exopolysaccharides

Cyanobacteria express high molecular weight glycans [extracellular polysaccharides (EPS)] with varying gelling abilities. The gelatinous form that occurs as a thin, firm fibrillar structure surrounding the cell wall, defining the shape of the cell, is called sheath, while the organized, densely packed nonuniform thick layer around the sheath that may/may not be tightly or covalently bound to the cell is called the capsule. Phospholipids covalently bound to the cell wall function as anchors. Attachment is through hydrogen bonds and hydrophobic and electrostatic interactions (Mayer et al. 1999; Wingender et al. 1999). Another fraction may exist loosely attached to the cell surface lacking definite margins or secreted in the environment, which is called slime or mucilage (De Philippis and Vincenzini 1998).

A particulate fraction corresponding to the transparent exopolymer particles (TEPs) has also been found to be associated with cyanobacteria (Thornton 2004).

Nearly 60% of the dry biomass may be composed of exopolysaccharides (Hill et al. 1997) that may be produced as a primary or secondary metabolite. In *Anabaena halophytica* (Sudo et al. 1995), *Spirulina platensis* (Filali Mouhim et al. 1993), and *Cyanospira capsulata* (Vincenzini et al. 1990), polysaccharide production parallels biomass production, while in *Cyanothece* BH68K (Fattom and Shilo 1984), *Nostoc calcicola* (Flaibani et al. 1989), *Phormidium* J-1 (Fattom and Shilo 1984, 1985), *A. flos-aquae* A37 (Moore and Tischer 1964; Tischer and Davis 1971), and *A. cylindrica* 10C (Lama et al. 1996), highest production rates were observed in the late phase. Conversely, in the case of a *Nostoc* strain, the highest rates of polysaccharide synthesis and release were achieved by young cultures (Mehta and Vaidya 1978). While most cyanobacteria produce heteroglycans, homopolysaccharide composed of α-D-1,6 glucose has been reported in a marine diazotrophic *Cyanothece* sp. (Chi et al. 2007). *Microcystis wesenbergii* represents another unique case where the polymer is exclusively composed of uronic acids (Forni et al. 1997). The heteroglycans, common to most cyanobacteria, are composed of 5–8 monomer repeats (Rossi and De Phillippis 2015); however, a decasaccharide repeating unit has been proposed for *Cyanospira capsulata* (Marra et al. 1990; Garrozo et al. 1995). EPS from *Spirulina platensis* and the thermophilic *Mastigocladus laminosus* have a still complex structure being composed of 15 monomer repeats (Filali Mouhim et al. 1993, Gloaguen et al. 1999). Fourteen monosaccharides have been reported to be present in *Chroococcus minutus* B 41.79 (Fischer et al. 1997). Rossi and De Philippis (2016) in their review on algal polysaccharides have listed the composition of 136 forms of cyanobacteria from various reports. Analysis of this data shows that eight different neutral sugars are generally present in various combinations and molar ratios, with glucose being the most prevalent sugar followed by galactose, fucose, mannose, arabinose, ribose, and fructose. In some cases, sugars such as xylose, galactose, arabinose, or fructose were found to be higher than glucose (Pereira et al. 2009). Pentoses are generally absent in other polysaccharides of prokaryotic origin (Sutherland 1994). The moiety protects the neighboring glycosidic bonds from the more common glycan hydrolases (Helm et al. 2000) and is partially responsible for the gelatinous consistency of the polysaccharide. Presence of either galacturonic acid or glucuronic acid or both in most cyanobacterial polysaccharides along with sulfates vest in negative charges and thus impart adhesivity to the macromolecule (De Philippis et al. 2000; Mancuso Nichols et al. 2005).

Sulfated sugars are involved in cell recognition and adhesion that are crucial in biofilm formation and complexation of metal ions (Tease and Walker 1987). They also provide stability over a range of temperature, pH, and salinity degrees (Arad and Levy-Ontman 2013). Sulfated polysaccharides have been shown to have numerous bioactivities of medicinal value. For cyanobacteria living in alkaline habitats like *Microcystis flos-aquae* C3–40, the polysaccharide capsule accumulates iron and manganese that are necessary for cyanobacterial growth but are relatively insoluble in aerobic alkaline conditions (Parker et al. 1996). Gehrke et al. (1998) showed that

iron species complexed by EPS allow bacteria to attach on pyrite and that Fe (III)-ions complexed by uronic acids in the EPS were needed to dissolve pyrite.

Uronic acid is a highly hydrophilic substance and contributes to the highly absorptive character of the EPS that can absorb over 95% water by weight (Decho 1994). This is critical for the survival of cyanobacteria through desiccation. Hydrophilic moieties provide minerals, nutrients, and water to the growing cell (Rossi et al. 2012a, b). Uronic acids are present in nearly 90% polymers and can reach up to 20–30% of the released polysaccharide dry weight (De Philippis et al. 2007; Laurienzo 2010). Polymers containing nosturonic acid or uronic acids with lactyl moieties play a pivotal role in the ability of organisms to survive extreme environments as in *Nostoc commune* DRH-1, a desiccation-tolerant cyanobacterium that can survive −400 MPa (0% humidity) for centuries (Potts 1994). Such functional groups act as a spacer arm or linker that aid adherence important for biofilm formation and act as molecular scaffolds for covalent attachment of UV-absorbing pigments and other antioxidative compounds. Lactyl-containing mannose monomers have been reported in *Cyanospira capsulata*, a filamentous heterocystous form that grows in saline lakes (Garozzo et al. 1998).

Cellulose, an insoluble polysaccharide of linear β-1,4-glucan, is present in the sheath, slime tubes, or EPS of *Oscillatoria* sp. UTEX 2435, *Oscillatoria princeps*, *Nostoc* sp. UTEX 2209, *Gloeocapsa* sp. UTEX L795, *Scytonema hofmanni* UTEX 2349, *Anabaena* sp. UTEX 2576, *Phormidium autumnale* UTEX 1580A, *Synechocystis* sp., *Nostoc* sp. PCC7120, *Crinalium epipsammum*, and *Synechococcus* 7002 (de Winder et al. 1990, Nobles et al. 2001; Zhao et al. 2015). Depending on the extent of inter- and intramolecular hydrogen bonding, cellulose exhibits varying degrees of crystallinity (O'Sullivan 1997). It could have roles in gliding motility of hormogonia, desiccation tolerance, nitrogen-fixing efficiency of heterocysts, enhancing viability of akinetes, or protection from UV light and could serve as a means of attachment to the host plant in the formation of symbiotic relationships (Matthysse 1983; Nobles et al. 2001). Synthesis of cellulose in cyanobacteria has been correlated to the presence of cellulose synthase gene *CesA* which has homology with the cellulose synthase in vascular plants. Cellulose occurs possibly as a laminated layer between the inner and outer membrane and is an important component of the extracellular glycocalyx in *Synechococcus* PCC7002 (Zhao et al. 2015). The thermophilic cyanobacterium *Thermosynechococcus vulcanus* undergoes cell aggregation in response to light stress under suboptimal temperatures induced by cellulose accumulation in the wall (Kawano et al. 2011).

Other rare monosaccharides identified in the EPS of cyanobacteria include methylated sugars, amino sugars like N-acetyl glucosamine, 2,3-O-methyl rhamnose, and acofriose as found in spirulan. 2-O-methyl D-xylose has been reported in the sheath of *Gloeothece* sp. PCC 6501 (Weckesser et al. 1987). N-acetyl fucosamine is found in large amounts in the arabinofucan EPS of *Synechocystis aquatilis* (Flamm and Blaschek 2014). Other methylated sugars like 4-O-methyl rhamnose and 3-O-methyl glucose have also been reported (Hu et al. 2003) (reviewed by Delattre et al. 2016). Methyl sugars perhaps play a role in certain recognition events (Staudacher 2012). The sugar moiety of EPS from *Wollea*

saccata was reported to have 60% hexoses and 31% 6-deoxyhexoses and 9% of pentoses with 40 types of methylated sugar derivatives suggestive of a very complex structure (Šutovská et al. 2017).

Certain cyanobacterial EPS contain peptides and ester-linked acetyl groups (up to 12% of EPS dry weight) (De Phillipis et al. 1998; Richert et al. 2005). These components along with deoxy-sugars like rhamnose and fucose confer hydrophobic character on the EPS affecting its rheological, emulsifying, and adhesive properties (Shepherd et al. 1995). Fattom and Shilo (1984) demonstrated that all benthic cyanobacteria are hydrophobic, while all planktonic forms are hydrophilic. Presence of cations was found to be necessary for the expression of hydrophobicity with divalent cations being more effective than monovalents. Multivalent ions induce gel formation (De Philippis et al. 1993). Metal ion sequestration or immobilization also protects the cells from its toxic species and at times provides for certain ions essential for growth. Some cyanobacteria are capable of modifying EPS from hydrophobic to hydrophilic character and can detach from surfaces as observed in *Phormidium* sp. when conditions become inappropriate (Fattom and Shilo 1985). Others have both hydrophilic and hydrophobic fractions that enable adhesion as well as water storage (Rossi et al. 2012a, b). Such amphiphilic exopolymers help stabilization of emulsions or act as flocculants (Fattom and Shilo 1985). Aggregation and flocculation of suspended particles by flocculants allow for light penetration to the sediment-water interface, thus facilitating survival and growth of benthic cyanobacteria that occupy a low-light zone. The flocculant may also carry nutrients to this zone (Bender et al. 1994; Fattom and Shilo 1984). Emulcyan, a sulfated heteropolysaccharide synthesized by *Phormidium* J-1, contains fatty acids and proteins that contribute variable degrees of hydrophobicity to the macromolecule (Bar-Or and Shilo 1987).

Adhesivity is an important character in mat formation and creating associations with plants as in *Nostoc* and wheat roots (Gantar et al. 1995) and symbiosis in *Anabaena azollae* (Robins et al. 1986). Polypeptides enriched with alanine, glycine, isoleucine, leucine, phenylalanine, and valine have been reported in the EPS of *Cyanospira capsulata* and *Nostoc calcicola* (Flaibani et al. 1989; Marra et al. 1990). *Schizothrix* sp. is a dominant cyanobacterium in the marine stromatolites found on the margins of Exuma Sound, Bahamas. The EPS released by this organism contains 2.5% protein, specifically enriched with aspartic and glutamic acid. These proteins act as nucleation centers for $CaCO_3$ precipitation. Changes in the EPS composition and stereochemistry lead to $CaCO_3$ polymorphisms (Kawaguchi and Decho 2002). The coccoid cyanobacterium *Solentia* (order Pleurocapsales) is an important component of stromatolite climax community that bores into the grains. The cell and its polysaccharidic sheath elongate as the cell divides and glides into the hole. Micrite composed of aragonite needles (<4 μm long) deposited on this sheath acts as a cement to form well-indurated layers (Reid et al. 2000; Dupraz et al. 2009). Other non-saccharidic components include phosphates, acetates, pyruvates, lipids, and DNA (De Philippis and Vincenzini 1998; Pereira et al. 2009).

The high number of different monosaccharides and their derivatives found in the cyanobacterial EPS, variety of substituent groups, linkages, and a broad range of possible macromolecular structures gives incalculable structural diversity and

functional variability to polysaccharides. According to a calculation by Werz et al. (2007), a trimer composed of 10 most frequently occurring mammalian monosaccharides alone may arrange in 126,000 possible combinations. With enormous versatility of their armor, cyanobacteria have an edge over other organisms against environmental stresses and thus occupy a special trophic status in the most extreme environments on earth.

EPS excretion serves multiple functions, including nutrient storage (organic compounds containing C, N, or P and trace metals), structural organization, and buffering against environmental stressors (Flemming and Wingender 2010). Studies on *Nostoc commune* have showed that EPS prevents membrane fusion, during periods of desiccation and subsequent rehydration. This, along with the synthesis of osmotica like trehalose and sucrose, may be the key mechanism in desiccation survival (Hill et al. 1997).

The highly hygroscopic EPS of *Chroococcidiopsis* maintains prolonged moisture around the cells, releasing it slowly (Caiola et al. 1996). A recent study on three strains of *Nostoc*, viz., *N. commune*, *N. verrucosum*, and *N. sphaericum*, that produce massive extracellular matrices showed that only EPS does not render desiccation tolerance. Expression of a 36kD Wsp A (water stress protein) and Sod F (superoxide dismutase) in *N. commune* was responsible for the tolerance, while the other strains without them were sensitive to desiccation (Sakamoto et al. 2018). Wsp A, perhaps, dynamically coordinates the flexibility and rigidity of the EPS matrix in response to desiccation-rehydration (Liu et al. 2017). The presence of capsules helps evade grazers as observed in *Phormidium* (Pajdak-Stós et al. 2001) and enhance nitrogen fixation by reducing oxygen permeability to nitrogenase as reported in the heterocysts of various species of *Nostoc* (Bergman et al. 1997; Soule et al. 2016) and in non-heterocystous forms like *Gloeothece* (Kallas et al. 1983). EPS of cyanobacteria also contains diverse phytochemicals. Presence of mycosporine-like amino acids in EPS has been reported by several authors. *Nostoc commune*, *Arthrospira platensis*, and *Microcoleus* sp. and *Leptolyngbya* sp. have been reported to actively secrete and accumulate them in their capsular polysaccharides (Bohm et al. 1995; Trabelsi et al. 2009, 2016). Radical scavengers have been reported in the EPS of *Anabaena* sp., *Tolypothrix tenuis*, *Phormidium*, *Nostoc*, *Oscillatoria*, and *Calothrix* (Parwani et al. 2014; Babić et al. 2015). The activity was attributed to the presence of phenolic acids, vitamin C, and flavonoids in *Leptolyngbya* (Trabelsi et al. 2016). *Anabaena* PCC 7120 and *Oscillatoria angustissima* have been reported to produce intra- as well as extracellular polysaccharides as a means of protection to toxic species (El-Sheekh et al. 2012). Cyanobacterial sheaths play an important role in enabling the microbe to survive environments subject to extensive mineralization. The sheath of *Calothrix* sp. was reportedly impermeable to particles sized $\geq$11 nm diameter, thus restricting silicification to the outer surface of the sheath preserving the cell wall and cytoplasmic functions (Phoenix et al. 2000; Benning and Mountain 2004). In natural environments, the complex EPS harbor numerous heterotrophic bacteria and undergo arrangement, rearrangement, dissociation, and resynthesis in a dynamic process buying time for acclimatization of the organism to changing

environment. It contributes to the structural stability of biofilms and mats, helps adhesion and attachment to substrate, and is implicated in cyanobacterial locomotion.

15.5.1 Transparent Exopolymeric Particles (TEPs)

Cyanobacterial exopolysaccharides vary in molecular structure depending on the producing species (Pereira et al. 2009). The sheaths of *Anabaena* C5 and *Nostoc* 2S9B have a sheetlike appearance, while *Anabaena* sheds its sheath by tearing off, leaving behind the nude filaments throughout the lifecycle, but the sheath of *Nostoc* is linked to hormogonia release which when liberated leave behind empty shells (Gantar et al. 1995). When the cell coating/mucilage detaches from the surface, it may further coagulate, gelate, or anneal to form submicron gels that further coagulate to form particulate (0.4–300 μm) TEP or colloidal TEP (0.05–0.4 μm) that can be visualized by Alcian blue staining. TEP can directly form from the fragmentation of capsules throughout the growth phase as observed in *Anabaena spiroides* or under nutrient limitation and on senescence following cellular lysis (Grossart et al. 1997; Berman and Viner-Mozzini 2001; Bittar and Vieira 2010; Verdugo and Santschi 2010; Berman-Frank et al. 2016) with dominance of the colloidal fraction (Villacorte et al. 2015). They can also develop abiotically by gelation, coagulation, or bubble adsorption (Chin et al. 1998; Passow 2000; Mari et al. 2017) under certain environmental conditions from dissolved fibrillar polysaccharides released from various planktonic organisms. TEPs exist as blobs, clouds, sheets, filaments, or clumps and have been detected in various aquatic ecosystems like rivers, lakes, groundwater, wastewater, brackish water, and seawater where they significantly contribute to the trophic structure, carbon cycling, and export nutrients to deep waters (Passow and Alldredge 1994; Passow 2000, 2002; Engel 2004; Berman-Frank et al. 2007).

The TEP macrogels (Verdugo et al. 2004) are composed of highly surface-active polysaccharides (Mopper et al. 1995) and thus have a strong tendency to form hydrogen bonds and bridge with ions like Na^{2+}, Ca^{2+}, and other metals. As a result, TEPs are usually extremely sticky, about two to four orders of magnitude stickier than phytoplankton or mineral particles with a high probability of attachment upon collision (Passow 2002; Engel 2004; Mari and Dam 2004; Liu et al. 2018).

Visible aggregates of TEP (>1 mm) have been reported in tank cultures of nutrient-depleted *Synechococcus* sp. (Deng et al. 2016) that sink at velocities of more than 400 m d^{-1} in seawater. *Microcystis* sp. has been reported to produce 15 pg Xanthan equivalents of TEP per cell (Liu et al. 2014). Interaction of this EPS with Ca^{2+} has been reported to induce colony formation in this bloom-forming cyanobacterium (Sato et al. 2017). *Crocosphaera*, a marine diazotrophic cyanobacterium, produces EPS and TEP constitutively during the exponential growth phase as has also been reported for *Anabaena flos-aquae* (Surosz et al. 2006; Sohm et al. 2011), while *Phaeocystis antarctica* produces them in stationary and death phase (Hong et al. 1997) and *Nostoc* under N limitation (Otero and Vincenzini 2004). Cyanobacterial blooms significantly contribute to the TEP pool (Bertocchi et al.

1990; Gloaguen et al. 1995) and are known TEP precursors (Passow 2000). Positive coupling between programmed cell death during bloom termination and Fe starvation and TEP production has been reported for *Trichodesmium* blooms (Berman-Frank et al. 2007). TEP concentrations reaching 1474 ± 226 μg xanthan gum equivalents L^{-1} have been reported in stationary phase cultures of *Prochlorococcus* sp., a picocyanobacterium-dominant primary producer in the oligotrophic ocean (Iuculano et al. 2017).

Because of their high abundance and unique properties, TEPs play a major role in the dynamics of the aquatic ecosystems. For example, as gel-like free swimming particles, TEP and TEP precursors show lectin-like property which can enable them to act as a chemical conditioning layer and to agglutinate bacteria (Li et al. 2015). It has been shown that about 0.5–25% of all bacteria present in seawater and freshwater were attached onto TEP. This suggests that free swimming TEPs are hotspots of intense microbial and chemical activity and act as a carrier to transport bacteria in aquatic environments. Evidence suggests that TEP can play an active role in the development of aquatic biofilms (Berman et al. 2011; Bar-Zeev et al. 2012) enhance surface biofouling and cycle nutrients vertically in deep waters (Passow 2002). Additionally, these particles together with their associated flora and fauna can serve as food packages for protists, microzooplankton, and even larval fish (Grossart et al. 1998). TEP-based aggregates or marine-snow containing TEP typically have high carbon (C)-nitrogen (N) ratios (Berman-Frank and Dubinsky 1999), which can also fuel N_2 fixation by heterotrophic diazotrophs (Rahav et al. 2013; Benavides et al. 2015).

15.5.2 Factors Affecting EPS Production

Composition of all structural and storage polysaccharides is more or less constant, yet EPS show a high amount of compositional flexibility. They also show a wide range of cellular N:P ratios, ranging from 5:1 to 100:1 depending on the type of nutrient that was in short supply, deviating a lot from the Redfield ratio of 106:16:1 (Geider and Roche 2002; Rabouille et al. 2017). This flexibility explains the capacity of these simple life forms to survive in nutrient extremes. The overconsumption of carbon is exuded as EPS. EPS production by phytoplankton is highly variable, from 1 to 99.9% of the net photosynthetically fixed organic carbon, depending on species and environmental conditions (Bertilsson and Jones 2003). Besides nutrient availability, other abiotic factors like light, temperature, pH, salinity, C:N ratio, nutrient source, batch or continuous cultivation, aeration, dilution, and availability of micronutrients also affect EPS production. Generally, exopolysaccharide production increases under stress, but what is stress to an organism may be a normal situation for another. Therefore, the responses are largely strain dependent.

An increase in EPS pool has been reported with increase in irradiance in *Crocosphaera watsonii* while the growth becomes saturated, and a similar response is observed at a low irradiance with nearly 30% of carbon occurring in TEPs (Rabouille et al. 2017). Increase in EPS with light intensity has also been reported in *Cyanothece* sp. (Su et al. 2007), *Aphanocapsa halophyta* MN11 (Matsunaga et al.

1996), *Gloeocapsa gelatinosa* (Raungsomboon et al. 2006), *Anabaena* ATCC 33047 (Moreno et al. 1998), and *Nostoc* sp. (Otero and Vincenzini 2003, 2004). The spectrum of energy also affects EPS productivity. Red and blue wavelengths were shown to enhance EPS production in *Nostoc flagelliforme* (Han et al. 2014) by altering carbon allocation and increasing carbon flow into the sugar nucleotide synthesis pathway (Han et al. 2018). Light was found to be the key factor in *Cyanothece* CCY0110 EPS production with a maximal yield being 1.77 gL^{-1} at 50 $\mu E\ m^{-2}\ s^{-1}$ (Mota et al. 2013). Light intensity and temperature have a synergistic effect (Carvalho et al. 2009). Temperature affects nutrient uptake, membrane fluidity, and photosynthetic rate and thus the EPS production. While a positive effect of temperature was observed on EPS production by *Anabaena* ATCC33047 (Moreno et al. 1998), no effect was observed in *Nostoc* sp. PCC 7936 (Otero and Vincenzini 2003, 2004).

Increase in salt concentrations increased EPS production in *Cyanothece* sp. ATCC51142, *Synechocystis* sp., *Spirulina*, and *Anabaena* PC1 (Nicolaus et al. 1999, Pereira et al. 2009; Ozturk and Aslim 2010), but *Cyanothece* CCY0110 being a marine form did not show much response (Mota et al. 2013). EPS content in *Synechococcus* strain CCAP1405 increases with salinity and age of cultures (Bemal and Anil 2018). *Spirulina subsalsa* showed a 2.5% increase in EPS in the stationary phase (Chakraborty et al. 2015) which suggests that nutrient starvation is needed to induce a response in this organism. The composition of the EPS also changes with a change in molar ratios of the monomers and composition.

Increase in C:N ratio has a critical role in EPS production. Usually, the presence of combined nitrogen even in diazotrophic forms enhanced EPS productivity perhaps because nitrogen fixation itself is an energy-intensive process (Kumar et al. 2007, Pereira et al. 2009). Reaction to N starvation is strain specific. An increase in EPS on N limitation has been reported in *Anacystis nidulan*s and *Microcoleus vaginatus* (Chen et al. 2006). A study on 15 *Cyanothece* species by De Philippis et al. (1998) showed that while a few strains showed an increase in intracellular carbohydrate, others showed increase in extracellular carbohydrate under N limitation. Response depended on the source of nitrogen in case of *Anabaena cylindrica* (Lama et al. 1996) and *A. flosaquae* (Tischer and Davis 1971). Excess nitrogen as nitrate generally does not affect significantly as it is the most easily metabolizable source. Urea was found to be the best nitrogen source for EPS production in *Nostoc flagelliforme* (Han et al. 2017). *Phormidium tenue* (Hu et al. 2003), *Spirulina subsalsa* (Chakraborty et al. 2015), and *Nostoc* sp. (Otero and Vincenzini 2003) showed an increase in EPS on N starvation, while no change was reported in *Synechocystis* (Panoff et al. 1988), *Cyanothece capsulata* (De Philippis et al. 1998), *Phormidium* (Fattom and Shilo 1984), and *Crocosphaera watsonii* (Sohm et al. 2011), and a negative effect was observed in *Phormidium laminosum* (Fresnedo and Serra 1992) (reviewed by Pereira et al. 2009).

Cade Menun and Paytan (2010) suggested a lower threshold value of phosphorous concentration at which carbohydrate accumulation is observed in *Spirulina platensis* (Markou et al. 2012). Increase in EPS in P starvation is reported in *Cyanothece* 16SOM-2 (De Philippis et al. 1993), *Synechococcus* sp. (Roux 1996),

Spirulina (Nicholaus et al. 1999), and *Anabaena* sp. (Huang et al. 2007), while no effect was observed in *Phormidium* J1 (Rossi and De Philippis 2016) and *Cyanospira capsulata* (De Phillipis et al. 1991), and a decrease has been reported in *Anabaena cylindrica* (Lama et al. 1996).

Concentration of divalent ions also affects EPS synthesis as observed in *Anabaena* sp. PCC7120 (Singh et al. 2016) in response to calcium chloride. High EPS production was observed at the inhibitory concentration of 10 mM, which suggests release of EPS as a means of chelation of the ion to protect the cell.

The composition of the EPS may vary with the age of the culture both quantitatively and qualitatively as observed for the sulfated polysaccharides produced by *Synechocystis* strains (Panoff et al. 1988) and *Spirulina platensis* PCC8005. *Spirulina* showed a decrease in the amount of galactose with culture aging, while *Synechocystis* showed variation in molar ratios, and one strain formed an additional polymer on aging (Filali Mouhim et al. 1993). On the other hand, the exopolysaccharide from *Cyanospira capsulata* showed no alteration in composition even after 10 years of cultivation (De Philippis and Vincenzini 1998). *Cyanothece* 16Som2 on continuous culturing for 5 years showed an additional sugar, rhamnose with variation in molar ratio in its EPS (De Philippis et al. 1998).

15.5.3 Rheological Behavior

Most cyanobacterial polysaccharides are polyelectrolytes. The charged groups ensure strong hydration. They may contain over 95% water by weight. A 20–40-fold increase in the weight of colonies of *Nostoc commune* was observed by Shaw et al. (2003) with most of it absorbed by the extracellular glycan. The EPS from *Anabaena* sp., *A. anomala*, *and A. oryzae* absorbs 25.9, 7.16, and 12.3 g H_2O g^{-1} polymer, while the polymer from *Tolypothrix tenuis* absorbs only 9.35 g (Bhatnagar et al. 2014b). Sacran absorbs an exorbitant amount of 6100 mL water per gram polysaccharide. The absorbing capacity is however dependent on the ionic strength of the solvent and decreases to 2700 ml in saline (Mitsumata 2018).

Polysaccharides do not form a true solution in water; however, on hydration, some of them undergo conformational transitions entering secondary, tertiary, and quaternary interactions (Rees 1982). These inter- and intramolecular interactions lead to characteristic hydrodynamic behavior such as viscoelasticity or gel-like properties. Viscoelastic behavior of EPS is responsible for the cell's mechanical integrity and is required for normal cell functioning, cellular homeostasis, cell-cell communication, stress response, and locomotive function (Bhat et al. 2012). An understanding of the flow behavior not only is relevant to industrial applicability of these polysaccharides but also gives an insight into the structure of the macromolecule. The viscosity and flow behavior (rheology) of the polysaccharides change in response to a number of variables, viz., the structure of the polysaccharide, size, concentration, temperature, pH, ionic strength, and shear. For Newtonian fluids, at constant temperature and pressure, viscosity does not vary with shear rate. On the other hand, for most non-Newtonian fluids, viscosity decreases with increase in

shear and are thus classified as pseudoplastic as against dilatant fluids that show increase in viscosity on increasing shear. Fluids that show increase in viscosity on constant shear with time are called rheopectic, while the ones that show a decrease are called thixotropic.

Cyanobacterial polysaccharides are characterized by high molecular weight (MW) that contributes to the viscosity which in certain cases is even greater than xanthan (Rossi and De Philippis 2015). *Cyanospira capsulata* has been reported to produce EPS with a molecular weight of 4.5 MDa, the highest reported so far. Table 15.3 summarizes some reported MW. Viscosities of cyanobacterial EPS may vary from as low as 0.9 cps as in *Nostoc calcicola* (Bhatnagar et al. 2014a) to 400 cps

Table 15.3 Molecular mass of cyanobacterial exopolysaccharides

Species	Apparent molecular mass (kDa)	References
A. circularis PCC 6720	>1200	Bar-Or and Shilo (1987)
A. halophytica GR02	2100	Morris et al. (2001)
Anabaena anomala	864	Bhatnagar et al. (2014b)
Anabaena circularis PCC 6720	41,200	Bar-Or & Shilo (1987)
Anabaena oryzae	539	Bhatnagar et al. (2014b)
Anabaena sp	3679	Bhatnagar et al. (2014b)
Anabaena sp. ATCC 33047	1350	Moreno et al. (2000)
Anabaena spiroides	2000	Colombo et al. (2004)
Aphanothece sacrum	1.6×10^4	Okajima et al. (2012)
Aphanothece stagnina	3.14×10^4	Le Nguyen et al. (2012)
Arthrospira platensis	81–98	Tseng and Zhao (1994)
C. capsulata ATCC 43193	1400–1900	Vincenzini et al. (1993)
C. minutus B 41.79	1200–1600	Fischer et al. (1997)
Cyanothece sp.	4.5×10^4	Ohki et al. (2014)
Gloeocystis vesiculosa	680	Halaj et al. (2018)
Microcoleus vaginatus	380	Hu et al. (2003)
Nostoc insulare 54.79	540–1300	Fischer et al. (1997)
Nostoc sp.	460	Hu et al. (2003)
Nostoc sphaeroids	131	Liu et al. (2018)
Oscillatoria sp.	200	Bender et al. (1994)
Phormidium versicolor NCC466 (CFv-PS)	63.79	Belhaj et al. (2018)
Phormidium J-1	1200	Bar-Or and Shilo (1987)
Phormidum 94a	2000	Vicente-Garcia et al. (2004)
Phormidum tenue	380	Hu et al. (2003)
Schizothrix sp.	300	Kawaguchi and Decho (2002)
Scytonema javanicum	110–380	Hu et al. (2003)
Tolypothrix tenuis	1953	Bhatnagar et al. (2014a, b)

in *Cyanothece* CE4 (De Philippis et al. 2001). EPS from *Nostoc calcicola*, a low-viscosity polymer (55–65 cps), showed a truly pseudoplastic, non-Newtonian, time-independent behavior with good recovery from shear (Bhatnagar et al. 2014a). Non-Newtonian shear-thinning properties have been reported for many other cyanobacteria also like *Spirulina platensis* (Filali Mouhim et al. 1993), *Anabaena halophytica* GRO2 EPS (Morris et al. 2001), *Cyanospira capsulata* (Lapasin et al. 1992), *Limnothrix redekei* (Moreno et al. 2000), *Anabaena variabilis* (Bhatnagar et al. 2012), *Nostoc carneum* (Hussain et al. 2015), and *Nostoc minutum* (Pereyra and Ferrari 2016). EPS from *Phormidium* 94a shows a Newtonian behavior at low EPS concentration changing to pseudoplastic above 0.1% solution and increasing hydration times perhaps due to increase in hydrogen bonding leading to a strong polymer network and viscosity (Vicente-Garcıa et al. 2004). Aqueous dispersions (0.1% w/v) of polysaccharide produced by *Cyanothece* strains were comparable to xanthan (De Philippis et al. 1998). Mancuso Nichols et al. (2009) screened 800 algal cultures for exosaccharide production and isolated the cyanobacterium *Microcystis aeruginosa* f. *flos-aquae* that showed highest viscosity (6.55 cps, equivalent to 1.16 g L^{-1} xanthan gum) in the medium. Parikh and Madamwar (2006) studied four cyanobacterial strains: *Cyanothece* sp., *Oscillatoria* sp., *Nostoc* sp., and *Nostoc carneum*. All the polysaccharides were low-viscosity products (6.9–18.4 cps) and showed decline in reduced viscosity with 0.1 M NaCl and precipitated with 0.1 M $CaCl_2$. A biphasic effect of metal ion concentration on the polysaccharide produced by *Microcystis flos-aquae* has been reported. The polysaccharide viscosity increased with increasing metal ion concentration ($CdC1_2$, $Pb(NO_3)_2$, $FeC1_3 > MnCl_2 > CuC1_2 > CaC1_2 >$ NaCl) reaching a maxima and then decreased with further addition of that ion (Parker et al. 1996).

Kinematic viscosity of *Nostoc* strains (*Nostoc commune*, *Nostoc flagelliforme*, and *Nostoc sphaeroides*) grown in the field was found to be higher than the suspension cultures grown under controlled condition (Huang et al. 1998). Apparent viscosity curves of EPS from *Arthrospira* sp. showed three phases. The first phase was characterized by Newtonian behavior at zero shear with viscosity reaching up to 10^2 Pa.s at 5% concentration. Beyond a critical shear value, the flow behavior became rheo-fluidifiant followed thereafter by another Newtonian region at a high shear rate (Chentir et al. 2017).

Polysaccharide properties are integrals of many factors. The primary structure of a polysaccharide is the main sequence of covalently linked sugar monomers. The constitutively fixed bond lengths and angles controlling the ring orientations comprise a secondary structure (configuration). In solution, polymer chains align themselves to adopt an orientation with lowest energy that may be ordered or disordered. Two general ordered conformations are ribbon-like and helix conformations. Polysaccharide with ribbon-like conformation is most easily aligned and closely packed through numerous hydrogen bonds and van der Waals forces. The resultant compact structures essentially prevent solvent penetration and retain insolubility in water. The ribbon-like conformation is the least soluble followed by the hollow helix, while polysaccharides with disordered conformation of a random coil are the most soluble. Stiff structure that hinders the intermolecular association remains extended and

usually leads to a higher solubility. Branched structure and presence of charged groups (carboxylate group, sulfate, or phosphate groups) increase solubility, while structural characters that promote the intermolecular association lead to poor solubility, such as linear chain, large molecular weight, and other regular structural characters. Zhang et al. (2007) reported the order of chain flexibility of glucan as (1,4) β > (1,3) α > (1,4) α > (1,3) β > (1–6) α > (1–6) β, while β glucans are inherently flexible. Besides molecular structure, concentration, degree of polymerization, polydispersity, solvent characteristics, and temperature also affect the polysaccharide conformation. In poor solvents, interactions of chain segments with themselves are favored resulting in aggregation. In good solvent, interactions between solvent and chain segments are favorable resulting in extended conformations and high solubility. Stability in aqueous environments can only be achieved when interchain and intrachain interactions are favorable. Therefore, two or more stranded associations of helices, of ribbons, or of helices with ribbons are found. These can be regarded as tertiary and higher levels of structure (Rees and Welsch 1977). Native polysaccharides can link up further to form three-dimensional networks resulting in gels that help maintain hydration and integrity of the cells. An increase in viscosity coincides with an increase in surface. The most extended conformation is the random coil and thus exposes more surface area than does a helix and a single helix exposes more than a double helix. With the structural complexity observed in cyanobacterial polysaccharides, an immensely wide variety of solution behaviors are expected. However, very few studies have been conducted. Since cyanobacterial polysaccharides are generally polyelectrolytes, their conformation depends on the ionic strength of the solvent and their concentrations. In very dilute salt-free solutions, these macromolecules thus tend to adopt an extended rod-like conformation; however, conformations ranging from rigid rod to random coils have been reported.

A rigid/extra-rigid rod-type conformation has been envisaged for the exopolysaccharide from *Aphanothece halophytica* GR02 (AH-EPS) (Morris et al. 2001). Polysaccharides extracted from four filamentous cyanobacteria, viz., *Microcoleus vaginatus*, *Scytonema javanicum*, *Phormidium tenue*, and *Nostoc* sp., show a conformation intermediate to a stiff rod and a random coil (stiff coil or a flexible rod) (Hokpusta et al. 2003), while EPS of *Anabaena* sp. ATCC 33047 takes up an intermediate structure between a random coil polysaccharide and a weak gel. Rheological studies on *Cyanospira capsulata* EPS show two different viscoelastic responses at sufficiently high concentrations and molecular weights (Cesàro et al. 1990; Garozzo et al. 1995, 1998). The solution conformation of the EPS is that of a random coil with moderate flexibility. As the concentration increases, overlapping and entanglement coupling occurs along with flickering interchain cross-reactions between semi-flexible segments creating order in the system. Further increase leads to formation of an entanglement network locally stabilized through specific non-covalent intermolecular interactions leading to a weak gel-like consistency (Cesàro et al. 1990; Navarini et al. 1992). The gelatinous EPS of *Nostoc commune* that grows in extreme conditions of desiccation is a biological gel that shows properties of both physical and chemical gels. The gel shows a reversible stress

softening behavior perhaps due to intensive physical crosslinking that makes it behave as an elastomer, limiting the relaxation of individual chains.

Sacran, a megamolecular suprapolysaccharide produced by *Aphanothece sacrum*, is an extremely high molecular weight (>1.6×10^7 g mol^{-1}) polysaccharide composed of five major monosaccharides (glucose, xylose, rhamnose, galactose, and mannose) (Okajima et al. 2009; Ohki et al. 2018). Sacran shows a very low overlap concentration of 0.004% indicating its megamolecular structure. The chains are not fully extended in pure water and take double-helical conformation at concentrations (c) >0.09 wt %, form a weak gel at c > 0.25 wt%, and finally form huge domains of liquid crystalline gels considered to be an aggregate of highly ordered helices, forming self-orienting micro-rods longer than 3 μm at c > 0.2 wt% (Mitsumata et al. 2013). During the drying process of the sacran solution, the rigid polysaccharides exhibit self-orientation and self-assemble to build a rod-like microdomain in micrometer scale (~1 μm of outer diameter and > 20 μm length) which have not been reported for any other soluble polysaccharides. Under certain conditions, clear twisting structures are formed (Okeyoshi et al. 2016; Budpud et al. 2018).

15.6 Industrial Applications

Cyanobacteria are gaining attention of the industry due to the ease of production with minimum cheap supplements, eco-friendly nature, and immense functional versatility that is difficult to reproduce synthetically. Though the productivity is lesser than other bacteria and fungi, their unique composition and interesting properties drive research in the field. Their potential for application in some areas is discussed here.

15.6.1 Lipopolysaccharides

Cyanobacterial lipopolysaccharides are generally considered as toxins and are attributed with a range of pathological effects. They can cause strong allergic reactions and skin and eye irritations and can induce symptoms of influenza like rigors, uneasiness, headaches, arthralgia, somnolence, marginal loss of memory, and diarrhea (Jakubowska and Szelag-Wasielewska 2015). However, cyanobacterial lipopolysaccharides are reported to be ten times less harmful than other bacterial variants. LPS from *Oscillatoria* sp. has been reported to activate cells of the immune system (Mayer et al. 2011, 2016; Ohkouchi et al. 2015). An exception was reported by Best et al. (2002) who investigated the potential of isolated cyanobacterial LPS to reduce the activity of glutathione S-transferases (GSTs) in zebra fish embryos which was found to be greater than LPS from *E. coli* or *Salmonella typhimurium*. Reduction in GST decreased utilization of glutathione, and glutathione depletion prevented LPS-induced inflammation as observed in case of lung injury (Nathens et al. 1998). It also has a protective effect on various models of apoptotic and necrotic liver injury

(Hentze et al. 1999, 2000). This property of cyanobacterial EPS has been proposed as a novel anti-inflammatory pharmacotherapy (Szászi et al. 2005; Stewart et al. 2006).

An LPS-related molecule derived from the cyanobacterium *Oscillatoria planktothrix* FP1, termed CyP, acts as a TLR4 receptor antagonist and blocks toxicity associated with other Gram-negative bacteria (Carillo et al. 2014; Swanson Mungerson et al. 2017). It acts as a competitive inhibitor of *Escherichia coli* LPS binding to the receptor complex on human dendritic cells (Macagno et al. 2006). Inhibition of cytokine production by Cyp in septicemia induced by *Neisseria meningitidis* in a human whole-blood model was reported by Jemmett et al. (2008) which thus can be considered as a new adjunctive therapy for treating septicemia. LPS preparations from *Oscillatoria planktothrix* sp. have also been proposed for the treatment and/or prevention of bacterial gum diseases primarily caused by *Actinobacillum actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, and, more importantly, *Porphyromonas gingivalis* that causes gingivitis and periodontitis (pyorrhea) (Molteni 2011). CyP actively inhibits the proinflammatory cytokines induced by LPS in vitro even when added several hours after LPS exposure (Macagno et al. 2006). Furthermore, the effect was not species specific since it was reportedly active in human, mouse, and porcine cells (Jemmett et al. 2008; Thorgersen et al. 2008). Thus, the potential of CyP can be exploited for the treatment of noninfectious diseases, in which detrimental TLR4-driven inflammatory processes induced by endogenous ligands play a pivotal role. TLR4 antagonism by CyP can help in delaying seizures and reducing recurrence in animal models of neurological and neurodegenerative diseases such as in epilepsy and models of amyotrophic lateral sclerosis and Alzheimer's diseases (Marosso et al. 2010; Iori et al. 2017; Molteni et al. 2016).

15.6.2 Exopolysaccharides

The immense structural variability in cyanobacterial exopolysaccharides manifests into functional versatility. Due to various sol-gel properties resident in these polysaccharides, they are variously used as thickening, emulsifying, gelling agents and stabilizers in food industry (Delattre et al. 2011; Kraan 2012). Xanthan is widely used in the food industry for its rheological behavior. EPS from *Cyanospira capsulata* and *Anabaena halophytica* GRO2 show xanthan-like physical properties (Cesàro et al. 1990; Navarini et al. 1990, 1992; Morris et al. 2001), while *Anabaena* sp. ATCC 33047 EPS is similar in properties to Alkemir 110 that is widely used in the food industry as a stabilizer (Moreno et al. 2000). *Microcystis flos-aquae* C3–40 resembles the plant polysaccharide pectin in its composition. Pectin is used as a gelling agent but requires intensive processing. Thus, the ease of preparation of the cyanobacterial polymer is a promising alternative. The exopolysaccharides of *Nostoc commune* are often used as a dietary ingredient in countries such as China and Peru (Johnson et al. 2008). These polymers have been suggested for applications as bioemulsifiers in cosmetics, swelling agents in food industry, and stabilizers in

textile and pharmaceutical industry. They can also be of use as industrial gums owing to their capacity to form weak gels (Parikh and Madamwar 2006).

Humectants that are commonly used in the cosmetic industry are glycerin, sodium pyrrolidone carboxylic acid, propylene glycol, and urea (Rawlings et al. 2004). These chemicals though have appreciable water absorption ability, and their retention ability is poor, thereby necessitating the use of occlusive agents to minimize transepidermal loss (Zhao et al. 2013) which may impart undesirable odor and greasy texture (Kraft and Lynde 2005). Though generally considered safe, they may trigger adverse skin reactions particularly in people with dermatitis (Zesch 1982). Cosmetic industry therefore has a demand for safer, nonirritant alternatives (Lodén et al. 2002). Amphipathic cyanobacterial exopolysaccharides trap water and protect live cells during periods of desiccation by retarding water loss (Tamaru et al. 2005). The exopolysaccharides of *Nostoc commune* exhibit a moisture absorption rate of 28% on exposure to 43% relative humidity for 24 h, which was much higher than that of chitosan (6.3%) and urea (5.8%) (Li et al. 2011). Sacran, a giant anionic polysaccharide extracted from the cyanobacterium *Aphanothece sacrum*, exhibits tenfold higher moisture retention than hyaluronic acid. This gummy polysaccharide consists of 11 different monosaccharides with ~12% carboxyl and ~11% sulfate groups per sugar chain (Okajima et al. 2008; Derikvand et al. 2017). Due to their excellent water-holding capacity, cyanobacterial EPS has great potential for being exploited as humectants in the skin care industry without the need of occlusive agents.

Another feature which makes cyanobacterial exopolysaccharides suitable for skin care is its antioxidant activity that, besides giving protection, also slows down the aging process. EPS capable of scavenging both superoxide anions and hydroxyl radicals in vitro (Li et al. 2011) can also mitigate oxidative damage induced by paraquat (Li et al. 2011). Sed et al. (2017) proposed extraction of exopolysaccharides for cosmetic use from spent culture systems of *Arthrospira platensis* that also exhibited antioxidant activity.

Cyanobacterial polysaccharides have also garnered interest in commercialization due to their potential in medicine. Scytonemin, a commercialized extracellular pigment present in the sheath of *Scytonema,* controls the cell cycle by regulating mitotic spindle formation and activity of kinases. It also inhibits proliferation of human endothelial and fibroblast cells (Stevenson et al. 2002). Polysaccharides from *Phormidium versicolor* (NCC466) protect liver tissues from cadmium toxicity (Belhaj et al. 2018). Consequent to their excellent biocompatibility, stability, efficacy, nontoxicity, biodegradability, low cost, and distinctive physicochemical properties, sulfated cyanobacterial polysaccharides can be used as nanocarriers for bioimaging and therapeutic applications (Radonić et al. 2010). Spirulan that exists as calcium (CaSp)/sodium spirulan (NaSp) is a sulfated polysaccharide prepared from *Arthrospira platensis*. It exhibits antithrombin activity by the activation of heparin cofactors (Hayakawa et al. 2003). Depolymerized NaSp can function as a precursor of the agents that prevent atherosclerosis as it acts as a potent inhibitor of arterial smooth muscle cell proliferation in vitro (Kaji et al. 2004) and selectively inhibits the entry of enveloped viruses and is reported to be active against HIV-1, HCMV, HSV-1, measles virus, mumps virus, and influenza A virus (Hayashi et al.

1996; Ayehuni et al. 1998; Hayashi 2008). TK V3 polysaccharide, another variant, was shown to inhibit replication of HIV, HCMV, HSV-1, human herpesvirus type 6 (HHV-6), and VACV, but not the enveloped viruses Epstein-Barr virus and influenza A virus (Kolender et al. 1997). Mansour et al. (2011) found that the polysaccharides isolated from *Gloeocapsa turgidus* and *Synechococcus cedrorum* had higher antiviral activity against rabies virus than that against herpes-1 virus. The exopolysaccharide from *Aphanothece halophytica* has antiviral activity against influenza virus A (H1N1), which shows 30% inhibition of pneumonia in infected mice (Zheng et al. 2006). Nostoflan from *Nostoc flagelliforme* shows antiviral activity against a variety of enveloped viruses whose cell receptors are carbohydrates such as influenza virus, herpes simplex virus-1, HSV-2, and human cytomegalovirus (Kanekiyo et al. 2005, 2007). EPS from *Nostoc commune* shows antimicrobial activity against *Escherichia coli*, *Bacillus anthracis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Serratia marcescens, Aspergillus niger,* and *Candida albicans* (Qian et al. 2012; Matsui et al. 2012; Liao et al. 2015; Li and Guo 2018). The polysaccharides from *Synechocystis* sp., *Gloeocapsa* sp., and *Nostoc entophytum* inhibit the growth of selected pathogenic bacteria and the fungus *Candida albicans* (Najdenski et al. 2013). *Phormidium versicolor* polysaccharides have been reported to be active against Gram-positive and Gram-negative bacteria as well as fungi (Belhaj et al. 2017). The elastomeric gel synthesized by *N. commune* can also be exploited for producing hydrogel films and scaffolds for tissue regeneration. The polysaccharides can also constitute scaffolds for tissue/organ regeneration in regenerative medicine (Nielsen et al. 2010; Kurd and Samavati 2015; Rodriguez et al. 2017).

Wounded skin exhibits a rise in the oxidant levels which can trigger chronicity of wounds especially in diabetic patients, and carcinogenesis and promote tumor progression via cell proliferation and cell death pathways. Reactive oxygen species (ROS) are also associated with various degenerative diseases; inflammation; and disorders such as cardiovascular disease, immune function decline, and aging (Rahman et al. 2012; Zhang et al. 2015). Nostoglycan reduces reactive oxygen species level and can suppress the proliferation of several types of tumor cells and induce apoptosis of human lung adenocarcinoma A549 cells via caspase-3 activation (Li et al. 2018). Spirulan inhibits pulmonary metastasis by preventing adhesion and proliferation of tumor cells (Mishima et al. 1998). Attempts are being made to prepare nanoformulations for commercialization against cancer (Bajpai et al. 2018). Potential of cyanobacterial polysaccharides in wound healing as a function of antioxidant activity has also been reported in *Anabaena anomala*, *A. variabilis*, *A. oryzae*, and *Tolypothrix tenuis* (Bhatnagar et al. 2014b). These hemostatic polymers were proposed to be used in the recovery from hemorrhagic wounds. Antioxidant activities have been reported in *Phormidium versicolor* (NCC 466) ECP also (Belhaj et al. 2017).

High molecular weight polysaccharidic preparation from the *Arthrospira*, called Immulina, has been commercialized as it exhibits significant immunostimulatory activity by raising TNFα, IFNγ, and IL-6 blood levels (Løbner et al. 2008; Nielsen et al. 2010). It is 100–1000 x more active as monocyte activation factor in vitro than

the polysaccharide preparations that were being used at the time in clinical settings for cancer immunotherapy (Løbner et al. 2008). Brevitoxin, another polysaccharide isolated from *Aphanizomenon flos-aquae* is reported to be immunostimulatory (Pugh et al. 2001).

The complex polysaccharide of *Wollea saccata* is antitussive and bronchodilatory with the effect being equal to or better than salbutamol but lesser than codeine (Šutovská et al. 2017). Antidiabetic activity in intracellular and extracellular polysaccharides has been reported in *Oscillatoria* sp., *Leptolyngbya* sp., *Pseudanabaena* sp., *Lyngbya* sp., *Coelastrella* sp., *Aphanothece* sp., *Synechococcus* sp., and *Chroococcus* sp. (Priatni et al. 2016). Sacran when applied topically shows reduced transepidermal water loss in dry skin human subjects and displays antiallergic effects similar to hydrocortisone and tacrolimus in animal experiments. It decreases the severity of atopic dermatitis (AD) skin lesions, itch, and sleep disorder in AD patients and thus may serve as an alternative adjuvant and therapeutic antiallergic agent (Motoyama et al. 2018). Heteropolysaccharides from *Phormidium versicolor* NCC466 (CFv-PS) displayed strong antioxidant and hepatoprotective activity against cadmium toxicity (Belhaj et al. 2018).

Another area of interest in cyanobacterial polysaccharides is nanoparticle synthesis. Silver nanoparticle synthesis with antibacterial activity has been reported in cell-free extracts of *Limnothrix* sp., *Anabaena* sp., *Synechocystis* sp., and *Nostoc commune* attributed to extracellular polysaccharides (Morsy et al. 2014; Patel et al. 2015). *Lyngbya majuscula* reduces gold to form nanoparticles. Nucleation occurs on the cell surface, and surface-active molecules are suggested to be involved in metal ion reduction and stabilization (Bakir et al. 2018).

Anionic polysaccharides rich in uronic acids can be developed as biosorbents for easy metal recovery. *Limnothrix* sp. KO05 and *Synechocystis* sp. PCC6803 EPS have been demonstrated to be instrumental in biosorbing cadmium (Haghigi et al. 2017; Shen et al. 2018). Preferential adsorption of uranium by functional groups of the marine unicellular cyanobacterium *Synechococcus elongatus* BDU130911 has been reported by Vijayaraghavan et al. (2018). Heterogels of sacran with polyvinyl alcohol have been explored for selective neodynium (rare earth metal) sorption (Okajima et al. 2010). Selectivity toward neodymium over other earth metals has also been reported in sacran-sepiolite composites (Alcantara et al. 2014). Bionanocomposite with sacran chains complexed with multiwall carbon nanotubes has been synthesized that form hardened hydrogel beads with metals and can be collected by electrophoresis for metal recovery (Okajima et al. 2013).

An exopolysaccharide with properties of a good hydrophobic dispersant, an excellent emulsifier, as well as a flocculant has been isolated from a strain of *Cyanothece epiphytica*. Its potential as a biolubricant with characteristics better than the conventional lubricant "grease" has been proposed for tribological applications (Borah et al. 2018). Halophilic cyanobacteria like *Cyanothece* sp. ATCC 51142, *Aphanocapsa halophytica*, and *Synechococcus* sp., producing copious amounts of EPS (Matsunaga et al. 1996; Moreno et al. 1998; Shah et al. 1999) can be relevant to oil recovery as they can decrease surface tension, thereby increasing solubility and mobility (Abed et al. 2009).

Adhesivity in cyanobacteria by virtue of the polysaccharidic sheath has always been viewed as a nuisance for their role in biofilm formation; however, their potential in wastewater remediation through turf scrubbing has been recognized and adopted by numerous companies like Hydromentia, BioProcess Algae, OneWater Inc., and Green Shift Corp. Biofilm formation as a source of biomass for biofuel production has also been recognized (Choudhary et al. 2017). A xanthan analogue excreted by the cyanobacteria CSIRO505 has been evaluated for its adhesive property and was described as fourfold effective for wood (maple) bonding (1.5 MPa shear strength) compared to commercial PVAc glue (Mancuso Nichols et al. 2009). Role of EPS as a molecular glue in photosynthetic algal microbial fuel cells, to generate electricity in a carbon neutral fashion, is also being explored. An electrogenic response to light has been observed from sheathed cyanobacteria (*Phormidium*, *Nostoc*, *Spirulina*, *Anabaena*, and *Lyngbya*) indicating that mucilaginous sheaths do not insulate or prevent electrogenic activity (Pisciotta et al. 2010). Further the role of EPS in direct electron transfer to the electrode and thus efficient energy production has been reported for the chlorophyte, *Scenedesmus* sp. SB1 (Angelaalincy et al. 2017), that may have analogy in cyanobacteria and still needs to be explored.

15.6.3 Glycogen

Glycogen extracted from natural sources is used in the cosmetics industry as an emollient and hydrating agent (Marchitto et al. 2010), as an antiaging agent in combination with a protein and a flavonoid (Mausner 1992), as a humectant (Jialun et al. 2018), and as a lubricant in ophthalmic solutions (Cavallo et al. 2002).

Monodisperse glycogen or phytoglycogen nanoparticles and their derivatives are polyfunctional additives suitable for use in aqueous- or alcohol-based pharmaceutical or food formulations (Korenevski et al. 2016), as rheological modifiers (including modulation of thixotropic behavior), stabilizers of organic and biological materials, and photostabilizers in sunscreens. Some of the products having glycogen as one component are Dermosaccharides® GY, Oxygen® complex LS 9641, and VitaplexTM LS 9799 by BSF; Amino-Glyco kviar, Bio-Hydractyl, Cobiodefender EMR, Glycoenergyzer, Hairdensyl complex, and Hydrotensyl complex by Cobiosa; Marine spheres by Chemir; and PhytoSpherix by Mirexus Biotechnologies (SpecialChem c2018).

Amphoteric glycogen hydrogels using phosphorylase-catalyzed enzymatic polymerization have been prepared for biomedical applications (Izawa et al. 2009, Kadokawa 2018). Hussain et al. (2018a,b) synthesized self-healing ultrastretchable glycogen hydrogels with good mechanical properties. Patra et al. (2016) synthesized stimuli-responsive glycogen/N isopropylamide hydrogels by free radical polymerization using ethylene glycol dimethylacrylate as a crosslinker for colon-specific delivery of ornidazole and 5-aminosalicylic acid. Russo et al. (2014) describe a high-quality slow-release pharmaceutical formulation made of glycogen and alginate. Monodisperse spherical hyperbranched nano-polysaccharidic glycogen

nanoballs have been synthesized by Takahashi et al. (2011) as a new building block for biomedical engineering and to act as chaperone in protein engineering. Though these preparations are resourced from other sources, cyanobacterial glycogen can also be used on similar lines.

Interest in glycogen metabolism in cyanobacteria as a promising alternative for biofuel production has also been explored. Möllers et al. (2014) demonstrated that cyanobacterial biomass could be used as an efficient feedstock for bioethanol production since it has simplified cell walls and glycogen as the main storage polymer which is far easier to mobilize than starch, the main storage polymer for eukaryotic algae.

Efflux engineering involving inactivation of pathways leading to glycogen synthesis has been tried in *Synechococcus* sp. PCC 7002, *S. elongatus* PCC7942, and *Synechocystis* sp. PCC 6803 wherein knocking out the enzymes necessary for glycogen polymerization led to increased leakage of nonspecific carbohydrates, organic acids, and a number of metabolites, including key intermediates of carbon metabolism and compatible solutes (Carrieri et al. 2012; Grundel et al. 2012; Hickman et al. 2013; Xu et al. 2013; Hays and Ducat 2015). *Synechococcus elongatus* UTEX 2973 (Syn2973), the fastest-growing cyanobacterium, appears to hold promise for the biofuel industry as this engineered strain can secrete 35.5 mg sucrose L^{-1} h^{-1} and accumulate glycogen at the rate of 0.75 g L^{-1} d^{-1} under nitrogen-replete conditions (Song et al. 2016). *Synechococcus* sp. PCC 7942 has also been genetically modified to secrete noncrystalline cellulose, which may be converted to ethanol by yeast fermentation (Nobles and Brown 2008) and *Synechococcus* sp. (Ducat et al. 2012). *Synechococcus* sp. PCC 7002 has been engineered to produce mannitol that gave a yield of 10% of cell dry weight and after genetic inactivation of glycogen the production of mannitol increased to 30% (Jacobsen and Frigaard 2014). Similarly, production of other chemicals such as isoprene in *Synechocystis* sp. PCC 6803 (Bentley et al. 2014) and lauric acid in *Synechococcus* sp. PCC 7002 (Work et al. 2015) has been attempted in glycogenless strains.

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Halocin Diversity Among Halophilic Archaea and Their Applications

16

Vijay Kumar and Santosh Kumar Tiwari

Abstract

Haloarchaea and their metabolites show unusual properties such as stability under extreme conditions and create special interest for the search of novel products. In recent years, studies on systematics have intensified and identified many new genera and species. Haloarchaea belonging to class Halobacteria are divided into three orders: Halobacteriales, Haloferacales, and Natrialbales consisting of more than 48 genera and 216 species. Haloarchaea and their metabolites are useful for various industrial applications. Halophilic proteins and enzymes produced by haloarchaea remain functional under high salt concentrations, extreme pH, and high temperature at which bacterial counterparts denature. These features make haloarchaea an attractive source of a wide variety of biotechnological products, such as retinal proteins, osmolytes, carotenoids, various hydrolytic enzymes, polyhydroxyalkanoates (PHAs), and exopolysaccharides. The biomolecules produced by haloarchaea have important role in manufacturing of bioplastics, photoelectric devices, artificial retinas, holograms, bioremediation process, and numerous other potential applications in biotechnology. Further study is more focused to develop a low-cost platform for low-cost downstream process with better yield at larger scale. Halocins are the proteinaceous antimicrobial proteins/peptides (AMPs) secreted by several members of haloarchaea. The production, purification, and characterization of halocins have been studied from various members of haloarchaea to understand the unique importance for their possible applications. The therapeutic potential of halocins needs more exploration to decipher an alternate to clinical antibiotics.

Keywords

Haloarchaea · Extremophiles · Carotenoids · Halocins · Purification · Antibiotics · Applications

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16.1 Introduction

Microbes are present everywhere in the nature, but halophilic or salt-loving microorganisms are found to exist in hypersaline environments. These environments are ubiquitous and spreading mainly due to irrigation, rainfall, and overuse of fresh water. Halophilic microorganisms may belong to either prokaryotes or eukaryotes. Among prokaryotes, halophilic bacteria usually require 3–15% NaCl, whereas halophilic Archaea (haloarchaea) require higher salinity (>12% NaCl). The requirement of salt concentration for optimum growth varies among different members of halophilic microorganisms (Oren 2002). Several classifications have been proposed for these halophiles, but most adopted classification was proposed by Kushner and Kamekura (1998). On the basis of requirement of NaCl concentration for optimum growth, halophilic microbes are divided into three categories: slight, moderate, and extreme halophiles that grow at 1–3%, 3–15%, and 15–30% NaCl concentrations, respectively. Non-halophiles are those which grow at less than 1% NaCl concentration. Few non-halophilic members that are able to tolerate the high NaCl concentrations are known as halotolerant microbes. Most of the haloarchaea contains isoprenoid derivative pigments in their cell membranes leading to various color pigmentations such as orange, red, purple, etc. (Oren and Rodríguez-Valera 2001). Therefore, the growth of haloarchaea in salt lake and salterns usually imparts pink or red coloration. However, few members of eukaryotes such as blue green algae, e.g., Dunaliella salina, also provide coloration to the hypersaline environments. The cell shapes of haloarchaea are reported to be diverse, such as rods, cocci, or disks, or have more unusual forms, such as triangular, squares, and pleomorphs, especially when cultured at higher temperatures or the lower NaCl concentrations needed for their optimal growth (DasSarma and Arora 2001; Hezayen et al. 2001). This chapter is mainly focused on the characteristics, taxonomy, and diversity of haloarchaea and their biomolecules, especially halocin, with their potential to be used for various purposes especially in the fields of industrial and medical sectors.

16.1.1 Habitat

Haloarchaea which belong to class Halobacteria exist in different types of habitat where salt concentration exceeds from the level of sea water (3.5% NaCl). Such types of environments have worldwide distribution of haloarchaea and include aquatic systems especially salt lakes and saline soils. The diversity of halophilic Archaea in salt lakes is well studied, whereas saline soils are least explored. Due to rainfall and irrigation in such types of environments, the salt concentration is decreased and affects the life of haloarchaea (Oren 2006). Haloarchaea have been isolated from different hypersaline habitats such as salt lake, solar salterns, salt deposits, and salt-fermented food products such as fish sauces, animal hides, etc. Some members of haloarchaea can even survive in salt crystals (Oren 2010). Several species of haloarchaea have been isolated from various traditionally used high salt-fermented foods. Metagenomic analysis also indicated that the presence of DNA into

the animal or human intestine may be due to the consumption of such types of food products (Lee 2013).

16.1.2 Characteristics of Haloarchaea

The morphological features of the haloarchaea are greatly affected by growth conditions in comparison to bacteria. The members of haloarchaea vary in cell size (0.8–13 μm) and cell shapes which usually have pleomorphic and sometimes elongated, round, disc, and trapezoid shapes. The cell shape and size are directly dependent on nutritional availability, salinity level, and ionic strength in the hypersaline environments. Haloarchaea are generally found to be Gram-negative except halococci. The members of genus *Natronococcus* have shown the Gram variable characteristics. Most of the species of *Haloferax* and *Haloarcula* are generally pleomorphic in nature (Oren et al. 2009). Most of the members are reported to be strict aerobes, but few are also able to survive under anaerobic condition by utilizing nitrate, arginine, dimethyl sulfoxide (DMSO), etc. present in their environment (Enache et al. 2007; Bonete et al. 2015).

Haloarchaea are usually resistant to most of the antibiotics such as penicillin, kanamycin, erythromycin, ampicillin, etc. that are found to be sensitive for various members of bacteria. However, haloarchaea are generally sensitive to the specific antibiotics such as novobiocin, a potent inhibitor of DNA gyrase, and bacitracin that interferes with the dephosphorylation of bactoprenol, a membrane carrier molecule, and also inhibits the lipid biosynthesis. Certain members such as *Halococcus saccharolyticus* are reported to be resistant to novobiocin. Non-coccoid haloarchaea are generally lysed in the presence of bile acid that binds with glycoprotein layer of the cell membrane leading to membrane disruption, and therefore, cell is lysed. The bile acid is usually used to differentiate halophilic Archaea and bacteria (Oren 2002).

Several members of haloarchaea are found to be pigmented due to the presence of carotenoid in their cell membrane and impart coloration to the hypersaline environments. The carotenoids are the hydrophobic proteinaceous biomolecules that act as protectant against damage from ultraviolet light radiation (Rodrigo-Baños et al. 2015). Carotenoids are divided into carotene and xanthophylls. Carotenes are composed of mainly hydrogen and carbon units and therefore also called carotenoid hydrocarbons, whereas xanthophylls are composed of oxygen in addition to carbon and hydrogen units and therefore called oxygenated carotenoids (Rivera and Canela-Garayoa 2012). Most of the members produce C_{50} carotenoid pigments such as bacterioruberin. Other pigments such as lycopene and phytoene are also found but in lower concentration (Rodrigo-Baños et al. 2015). The level of salinity in hypersaline environment affects the production of pigment. The *Haloferax* species, e.g., *Haloferax mediterranei*, become more pigmented at lower salinity, whereas higher salinity (>25% NaCl) reduced the pigmentation to colorless due to lesser production of carotenoid pigments (Oren 2006).

Members of haloarchaea usually contain mainly four types of retinal pigments like bacteriorhodopsin, halorhodopsin, and sensory rhodopsins I and II into their cell membrane. Bacteriorhodopsin is a 27 kDa transmembrane protein which captures green light (500–570 nm) and generates proton gradient by expelling H^+ outside the cell leading to chemical energy with the formation of ATP molecules. Therefore, haloarchaea containing bacteriorhodopsin are able to grow during low nutrients availability in their environments by using light as an energy source. Second retinal pigment is the halorhodopsin, a transmembrane protein present in the cell membrane of haloarchaea. It absorbs the green or yellow light and uptakes the halide ions (Cl^-) inside the cell to maintain the osmotic equilibrium. The structure of halorhodopsin was initially elucidated from *Halobacterium salinarum.* The properties of both bacteriorhodopsin and halorhodopsin are similar and they transport the cations and anions in opposite direction (Essen 2002). Two sensory rhodopsins (I and II) are the phototaxis receptors, and transmembrane proteins have been studied in *Halobacterium salinarum* and *Natronomonas pharaonis.* Type I absorbs the green light, whereas type II absorbs the blue light and regulates the movement of flagella (Spudich and Luecke 2002; Klare et al. 2008).

Several members of haloarchaea contain flagella which is functionally similar and structurally different to bacterial flagella. It is made up of protein subunit known as flagellin (Syutkin et al. 2014). *Halobacterium salinarum* and *Haloferax mediterranei* produce the gas vesicles inside the cells. The production of gas vesicles is helpful for strictly aerobic halophilic Archaea. Gas vesicles are formed from proteinaceous components filled with air and help in providing buoyancy to the cells. Therefore, haloarchaea cells float to the surface of water to overcome the oxygen stress in hypersaline lakes (Pfeifer 2015). Cell membrane of haloarchaea contains a variety of lipids with ether linkage between isoprenoid hydrocarbon side chains and *sn*-glycerol-1-phosphate backbone, whereas ester linkage between fatty acid and *sn*-glycerol-3-phosphate is found in the case of Bacteria and Eukarya (Jain et al. 2014).

16.1.3 Factors Affecting Growth of Haloarchaea

Salt concentration, pH, and temperature are the major factors responsible for the growth of haloarchaea in hypersaline environments. The concentration of salt determines the distribution of haloarchaea species. Many members of haloarchaea grow at or above 15–18% NaCl concentration. Their cells are irreversibly damaged and lysed at less than 5–12% NaCl. In addition to NaCl, the levels of divalent cations such as Mg^{2+} and Ca^{2+} are also very important particularly for moderately halophilic Archaea such as *Haloferax* species growing in such types of saline lake. Haloarchaea are adapted to survive in wider pH range from slight acidic, neutral to alkaline. Most of the members isolated from different habitats are either neutrophilic or alkaliphilic, whereas acidophilic haloarchaea are rather limited. The temperature requirement for

growth of aerobic haloarchaea of the order Halobacteriales varies between 35 and 50 °C and sometimes even higher. Few members are able to tolerate the temperature as low as 0–11 °C and as high as 50–60 °C. Such types of variation depend on seasonal and geographical regions (Oren 2006, 2014).

16.1.4 Adaptation of Haloarchaea Under Hypersaline Environments

Haloarchaea are adapted to survive in moderate to hypersaline environments. The minimum requirement of salt for their growth varies among different members from 10 to 20% NaCl concentration. However, several members are able to grow in extreme salinity conditions containing 30–35% NaCl or near to salt saturation limit in salt lake. The various members have been isolated from crystalline salt in which they survive for a long period of time (Mormile et al. 2003). As the salinity level decreases in the hypersaline environments due to various natural events, the morphology of cells and protein function are affected leading to cell death of various microbes which are unable to tolerate such types of stress. In hypersaline environments, various stress conditions such as high temperature, pH, and less oxygen solubility in addition to higher salinity exist leading to limitation in growth of other microbes (Oren 2010). To cope with the osmotic stress in hypersaline environment, the strategy "salt-in-cytoplasm" is used by haloarchaea during which salts such as NaCl or KCl accumulate in the cytoplasm at concentrations similar to the extracellular ones (Oren 2013). Haloarchaea uptake the K^+ from outside to inside the cells and maintain the osmotic balance with extracellular Na^+. Therefore, haloarchaea keep the higher ionic strength inside the cells which stabilize the various halophilic proteins that require counter ions for their excess amino acids on their surface. In addition to potassium ions, various other cations such as Mg^{2+} or Ca^{2+} ions and solutes, e.g., glycerol, are also responsible for the stability and function of halophilic proteins produced by haloarchaea (Madern et al. 2000). Haloarchaeal DNA contained high G + C content that stabilizes in the presence of high cation concentrations into the cytoplasm (Lynch et al. 2012). Another mechanism to cope with osmotic stress is the accumulation of organic molecules such as glycine betaine, ectoine, and trehalose known as osmolytes. Compatible solutes or osmolytes are small and highly soluble molecules that can be accumulated at high concentrations inside cells without causing any harmful effects (Oren 2013). Haloarchaeal proteins are generally glycosylated which is responsible to cope against high salinity. Glycosylation of the S-layer in haloarchaea enables them to grow in media containing high salt concentrations and protects them against various proteases. The salinity of hypersaline environment determines the type of sugars involved in N-glycosylation of the S-layer and the position in which these sugars are added to this glycoprotein. Further study on protein glycosylation in haloarchaea is required to explore the role of glycosylation in the stability of halophilic proteins (Guan et al. 2012; Jarrell et al. 2014). Therefore, haloarchaea are able to survive in such types of extreme conditions which are generally lethal for other microbes (Burg and Ferraris 2008).

16.2 Diversity of Halophilic Archaea

The prokaryotic domains Bacteria and Archaea are categorized into 35 phyla (LPSN 2016). Domain Bacteria are subdivided into 30 phyla, whereas domain Archaea are subdivided into 5 phyla. The five phyla of domain Archaea are Crenarchaeota, Euryarchaeota, Korarchaeota, Nanoarchaeota, and Thaumarchaeota (LPSN 2016). The phylum Euryarchaeota is divided into eight classes: Methanomicrobia, Archaeoglobi, Halobacteria, Methanobacteria, Methanococci/Methanothermea, Methanopyri, Thermococci/Protoarchaea, and Thermoplasmata. Initially, Gibbons had described two genera and three species within family Halobacteriaceae (Gibbons 1974). Oren et al. (1997) described the polyphasic approach to identify a new isolate of haloarchaea. It includes information regarding cellular structure, different parameters for growth conditions, and chemotaxonomic features including polar lipid analysis of whole organism methanolysates and nucleic acid sequence data. Species-level identification of new isolates using 16S rDNA amplification and sequencing and DNA-DNA hybridization data further validate the physiological and biochemical characteristics and exactly determine the phylogenetic position with their relative taxa (Oren et al. 1997). The isolation and identification of various members of haloarchaea on the basis of polyphasic approach created the database that includes the diverse members of neutrophilic to alkaliphilic haloarchaea. Initially, the polar lipid analysis was used to identify the haloarchaea up to genus level, but recent study has shown the presence of certain glycolipids in more than one genera. Members of single genus have showed the different types of polar lipids that raise a controversy for their identification with polar lipid analysis and reduced the importance (Oren et al. 1997; Kamekura and Kates 1999). Till 2011, the family encompassed 129 species, classified based on a polyphasic approach, whose names have been validly published and classified in 36 genera under single order Halobacteriales (Oren 2012). Till 2014, 40 genera and 158 species were explored and studied. The type strains of each genus were from different environmental sources: water and sediment from hypersaline lakes (39%), water from solar salterns (22%), soils and rocks (16%), food (15%), and other sources (8%). Till 2015, all haloarchaea members were classified in class Halobacteria that was divided into single order Halobacteriales and single family Halobacteriaceae (Gupta et al. 2015). A large diversity of halophilic Archaea has been determined by both culture-dependent and culture-independent methods (DasSarma and Arora 2001), leading to a continuous increase in the number of known haloarchaeal genera and species.

Recently, conserved signature proteins and conserved signature insertions/ deletions have been introduced as new phylogenetic markers for haloarchaeal classification. According to this classification, the class Halobacteria, within the phylum Euryarchaeota, is divided into three orders: Halobacteriales, Haloferacales, and Natrialbales (Gupta et al. 2015). Currently, known species and genera belonging to the haloarchaea and the phenotypic characteristics of the type strain are described by Amoozegar et al. (2017). The order Halobacteriales contains three families: Halobacteriaceae (12 genera, 36 species), Haloarculaceae (8 genera, 30 species), and Halococcaceae (1 genera, 9 species). The order Haloferacales contains two

families: Halorubraceae (7 genera, 50 species) and Haloferacaceae (8 genera, 37 species). The order Natrialbales contains only one family, Natrialbaceae (12 genera, 54 species). The classification of haloarchaea from class to genus/species levels is depicted in Fig. 16.1. The genera *Natribaculum*, *Halosiccatus*, *Halocalculus*, and *Halovarius* have not yet been categorized according to the above mentioned phylogenetic markers.

Most of the members of haloarchaea are studied from salt lakes or salterns. However, screening methods utilizing several new compositions of culture media help in continuous identification of new strains. It has been reported that halophilic Archaea exist in various hypersaline environments such as salt lake, pools, mine, marine solar saltern, low-salt spring, saline soil, commercial salt, seawater aquarium, saltern sediment, saltern crystallizer, alkaline lake brine, Dead Sea, Red Sea, microbial mats, saline soda lake, salted hides, fermented food, and fish sauce. The new species has been reported from various hypersaline regions located in different countries mainly China, Spain, USA, Austria, Australia, Egypt, Korea, Japan, Iran, Thailand, Indonesia, Russia, Argentina, Kenya, Mexico, Tibet, France, Poland, Philippines, Taiwan, Romania, and Africa (Amoozegar et al. 2017).

Most of the members of class Halobacteria are aerobic, generally red-pigmented halophiles and strictly dependent on high salt concentrations for maintaining growth and cellular integrity (Grant et al. 2001). The representatives of haloarchaea are neutrophilic, many are alkaliphilic, and a moderately acidophilic species such as *Halarchaeum acidiphilum* isolated from commercial solar salt does not grow above pH 6.0 (Minegishi et al. 2010). Although scarce reports recorded the presence of Halobacteriaceae at relatively low salinities (Elshahed et al. 2004; Purdy et al. 2004), most species grow optimally above a concentration of 150 g/L salt and lyse at concentrations below 100 g/L (Oren 2011).

Hypersaline environment has been reported to maintain a high density of halophilic bacteria which belong to phyla *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*, and haloarchaea belong to phylum Euryarchaeota (Keshri et al. 2013; Fernandez et al. 2014). Various strains have been identified that belong to genera *Halobacterium*, *Halorubrum*, *Halarchaeum*, *Halomicrobium*, *Haloarcula*, *Halonotius*, *Natromonas*, and *Salarchaeum* isolated from salts (Chang et al. 2008; Roh et al. 2010). Using metagenomic analysis, the diversity of haloarchaea in solar salterns from different geographical locations was observed that demonstrated the presence of various members of *Halorubrum* and *Haloquadratum* genera, whereas culture-based methods demonstrated the presence of different strains mainly belonging to *Haloarcula*, *Haloferax, Halobacterium*, and *Halorubrum* genera (Burns et al. 2004; Bidle et al. 2005; Birbir et al. 2007; Oh et al. 2010; Zafrilla et al. 2010).

Several members of haloarchaea have been reported from salted and fermented food products. *Halobacterium* and *Natrinema* were reported in salt-fermented fishery products, and *Haloarcula marismortui* was isolated from salted anchovies (Moschetti et al. 2006; Tapingkae et al. 2010). Strains belonging to *Halococcus*, *Halobiforma*, *Halosimplex*, *Natrialba*, and *Natronococcus* genera have been isolated from “kimchi” – Korean fermented food (Chang et al. 2008). Five new strains such as *Haladaptatus cibarius*, *Haloterrigena jeotgali*, *Halakalicoccus*

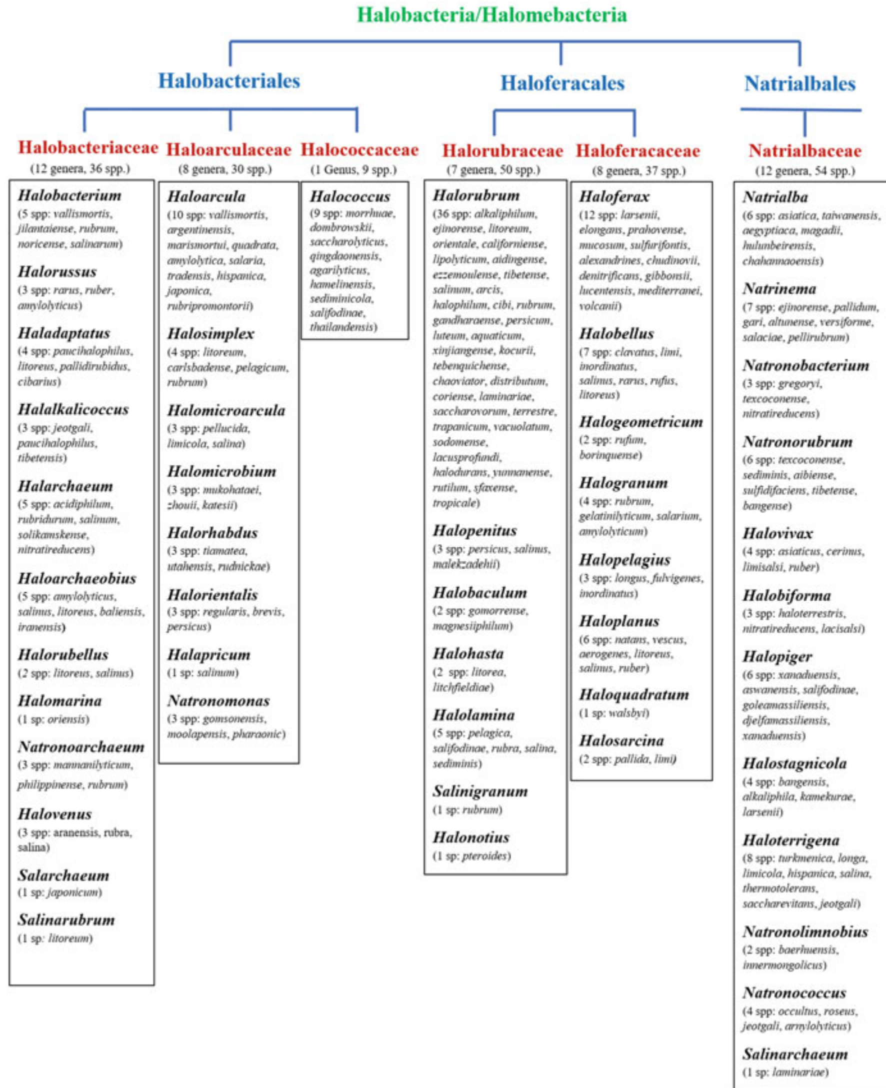

Fig. 16.1 Phylogenetic classification of haloarchaea from class to genus/species level

jeotgali, *Halorubrum cibi*, and *Natronococcus jeotgali* have been isolated from "Jeotgal" – Japanese salted-fermented seafoods (Roh and Bae 2009; Lee 2013). The salt itself has proven to contain viable microbial cells. In the process of salt crystallization, most haloarchaea present in the salterns are trapped inside fluid inclusions (Norton and Grant 1988). Most of the strains isolated from salt inclusions were related to *Halorubrum*, *Halobacterium*, *Haloarcula*, and *Halococcus* (McGenity et al. 2000; Mormile et al. 2003). Long-term survival of isolates related

to *Halorubrum*, *Natronomonas*, and *Haloterrigena* inside salt crystals was suggested to exceed 22,000 years (Schubert et al. 2010).

Halophilic Archaea have been identified by culture-dependent methods from foods consumed by people. The salts are being used since a very long time for the preservation of foods. The existence of haloarchaea in salted foods may be due to salterns where they grow (Kim et al. 2011; Shimane et al. 2011; Shimoshige et al. 2013). New types of haloarchaea have been discovered from fermented fish and fish sauces, and such studies are being conducted mostly in Korea, Japan, and Southeast Asian countries, especially Thailand. Korean people consume traditional foods including soy-fermented products, such as soy sauce and soybean paste, and fermented seafoods, such as jeotgal (fermented small fish or shellfish) and sikhae (fermented fish with salt and starchy grains). Southeast Asians (80–90%) consume fish sauce (Akolkar et al. 2010; Lee 2013), whereas Korean and Japanese normally use soy sauce for fermented condiments. One hundred fifty-six species of halophilic Archaea have been identified from various fish sauce products, and new species of halophilic archaea are continuously being reported (Tapingkae et al. 2010). Halophilic Archaea remain alive in salts after being heated by the sun in salterns. Halophilic Archaea isolated from fermented foods and salts are *Haladaptatus cibarius* from fermented shellfish (Korea); *Halalkalicoccus jeotgali* from shrimp jeotgal (Korea); *Halogranum salarium* from sea salt (Korea); *Halorubrum cibi* from shrimp jeotgal (Korea); *Haloterrigena jeotgali* from shrimp jeotgal (Korea); *Natronococcus jeotgali* from shrimp jeotgal (Korea); *Halarchaeum acidiphilum*, *Halostagnicola alkaliphila*, *Halobaculum magnesiiphilum*, *Natronoarchaeum mannanilyticum*, and *Salarchaeum japonicum* from commercial salt (Japan); *Haloarcula tradensis*, *Haloarcula salaria*, *Halococcus thailandensis*, and *Natrinema gari* from Thai fish sauce, namely, nam-pla and *Halobacterium salinarum* from fermented fish (pla-ra) in Thailand. Few acidophilic haloarchaea like *Salarchaeum japonicum* and *Halarchaeum acidiphilum* and alkaliphilic haloarchaea like *Natronoarchaeum mannanilyticum* (Shimane et al. 2011) show diverse pH-dependent characteristics. The haloarchaea strains isolated from various food products, solar salterns and salt lakes demonstrated diversity among various strains. Due to the lack of attempts to design new screening media, various members of halophilic Archaea still remain unexplored. Archaea belonging to the genus *Halorubrum* may be present in various food products.

Although culture-dependent methods have been used to analyze microflora within foods or other hypersaline environments, the conventional methods are helpful for the identification of a limited number of strains. Therefore, culture-independent techniques such as PCR and denaturing gradient gel electrophoresis (PCR-DGGE), library cloning, restriction fragment length polymorphism (RFLP), or next-generation sequencing (NGS) are rapidly being developed for analyzing microflora or microbiomes (Vitali et al. 2010). Halophilic Archaea isolated from foods has also been identified using culture-independent techniques such as PCR-DGGE and barcoded pyrosequencing (NGS methods) and quantitative real-

time PCR (qPCR) has been reported. *Halovivax ruber* and other strains were identified from different types of jeotgals (Roh et al. 2010). Furthermore, Park et al. used qPCR to enumerate the changes of archaea during the fermentation process of kimchi (Park et al. 2009), changes of PCR-DGGE profile as fermentation progressed have been studied, and a large number of strains such as *Halococcus* spp., *Natronococcus* spp., *Natrialba* spp., and *Haloterrigena* spp. were reported. Barcoded pyrosequencing study showed that 68.6–98.4% of strains of the phylum Euryarchaeota in jeotgal belonged to the family Halobacteriaceae, among which most were belonging to *Halorubrum* and *Halalkalicoccus* genera (Chang et al. 2008).

The phylogenetic diversity and community structure of members of the Halobacteriales have been investigated in various thalasohaline, e.g., crystallizer ponds in solar salterns (Maturrano et al. 2006; Baati et al. 2010; Oh et al. 2010), and athalasohaline, e.g., the Dead Sea and soda lake water bodies (Oren 2010). Furthermore, the number of microbiomes that live inside the human body is extremely larger, reaching approximately 10^{13}–10^{14} CFU, and the size of their genome is close to 100 times bigger than that of the human genome (Gill et al. 2006), although more than 90% of these enteric microorganisms are bacteria that belong to divisions of *Bacteroidetes* and *Firmicutes*. Unlike bacteria, the number of Archaea among the enteric microbiomes is very small, mostly methanogenic Archaea. It is very interesting to note that the diversity is quite low for methanogenic Archaea inside the intestines since most of them belong to the three species *Methanobrevibacter smithii*, *Methanosphaera stadtmanae*, and *Methanobrevibacter arboriphilus*, whereas, on the other hand, enteric halophilic Archaea have a relatively greater variety, mostly belonging to the genus *Halorubrum*. Hypersaline environments are inhabited by a great variety of microorganisms (Samuel et al. 2007; Oxley et al. 2010).

16.3 Halocins

Halocins are the proteinaceous antimicrobial proteins/peptides (AMPs) secreted by several members of haloarchaea belonging to class Halobacteria. Most of the haloarchaea produce optimum halocin during transition from exponential to stationary phase. The exception is halocin H1 whose production is found to be maximum at mid-exponential phase and remains stable till the stationary phase (Shand and Leyva 2007; Besse et al. 2015). Few members of haloarchaea, e.g., *Haloferax mediterranei*, show a decline in their halocin level during the stationary phase. Hence, the production of halocin is found to be growth associated, and it can be used as model to study phase-dependent halocin expression (Cheung et al. 1997; Price and Shand 2000). On the basis of antagonism study, hundreds of different types of halocins are expected to be present in nature but few halocins have been characterized till date (Ghanmi et al. 2016). The biochemical properties of different

halocins are summarized in Table 16.1, and the recent updates about halocins (producers belonging to phylum Euryarchaeota) and sulfolobicins (producers belonging to phylum Crenarchaeota) are described.

1. **Halocin H4:** Halocin H4 is the first studied halocin produced from *Haloferax mediterranei* R4 (ATCC33500), isolated from a Spanish solar salt pond in Alicante, Spain (Rodriguez-valera et al. 1981; Meseguer and Rodriguez-valera 1985). The molecular weight of halocin H4 is 39.6 kDa. The activity was initially detected as the culture began its transition into the stationary phase which is salt-dependent and sensitive to proteases and high temperature. The antimicrobial activity of halocin H4 disappears at 60 °C (for 24 h), 70 °C (for 4 h), and 80 °C (for 30 min) (O'Connor and Shand 2002). The halocin activity remains stable at above 15% NaCl (Rodriguez-valera et al. 1982). Halocin H4 inhibits many members of the haloarchaea such as *Halobacterium salinarium* CCM2090 and exhibits several features of halophilic proteins such as high content of negatively charged amino acids, especially aspartate, low content of lysine, and high content of non-bulky residues (proline, glycine, valine, and threonine). It possesses hydrophobic 32-amino acid fragments (residues 178–209) (Cheung et al. 1997).
2. **Halocin H1:** Halocin H1 was purified from *Haloferax mediterranei* strain M2a (previously Xia3) isolated from salt ponds in Santa Pola (Alicante, Spain) (Rodriguez-Valera et al. 1981; Platas et al. 2002). The molecular weight of halocin H1 is 31 kDa. Optimum production of halocin H1 occurs when the culture enters into the stationary phase grown at 20% NaCl, 37 °C, and 220 rpm. The halocin activity remains stable in the stationary phase. Like halocin H4, the antimicrobial activity of halocin H1 is sensitive to higher temperature and salt-dependent. Halocin H1 is stable up to 50 °C only and requires the minimum 6% NaCl concentration for activity (O'Connor and Shand 2002; Platas et al. 2002). Dialysis of CFS against lower salt concentrations caused an immediate decrease in its activity and cannot be restored by dialysis against the initial saline conditions. The activity was completely reduced within 7 days after dialyzing against water (Platas et al. 2002). The desalting effect irreversibly denatures halocin activity which may be due to perturbation in three-dimensional structure of halocin H1 (Torreblanca et al. 1989; Platas et al. 2002; Pasic et al. 2008; Kavitha et al. 2011). Most of the properties of halocin H1 are common to halocin H4 such as both are produced from *Haloferax mediterranei* isolated from Spanish salterns and their sizes are above 30 kDa. Both producer strains are not able to inhibit each other. All these properties of both halocins suggested that both of them are related or might even be the same halocin (Shand and Leyva 2007). It inhibited the different members of the haloarchaea. Halocin H1 was not inhibited the growth of halotolerant eubacteria but demonstrated a wide range of antimicrobial spectrum against haloarchaeal strains (Platas et al. 2002). The

Table 16.1 Biochemical properties of halocins produced by different strains of haloarchaea

S. No.	Halocin	Producer	Source	Properties (Molecular weight; thermostability; salt dependence; host range; protease sensitivity; mode of action)	References
1	A4	Strain TuA4	Solar saltern, Tunisia	7435 Da; >100 °C; no; broad; ND; ND	Haseltine et al. (2001)
2	C8	*Halobacterium* strain AS7092	Great Chaidan salt Lake, China	6.3 kDa; >100 °C; no; broad; proteinase K; ND	Li et al. (2003)
3	G1	*Halobacterium* strain GRB	Solar saltern, France	ND; ND; ND; broad; ND; ND	Sun et al. (2005)
4	H1	*Haloferax mediterranei* M2a (or Xai3)	Solar saltern, Alicante, Spain	31 kDa; <50 °C; yes; broad; ND; membrane permeability	Soppa and Oesterhelt (1989)
5	H2	Haloarchaeon Gla2.2	Solar saltern, Alicante, Spain	ND; ND; ND; broad; ND; ND	Platas et al. (2002)
6	H3	Haloarchaeon Gaa12	Solar saltern, Alicante, Spain	ND; ND; ND; broad; ND; ND	Rodriguez-Valera et al. (1982)
7	H4	*Haloferax mediterranei* R4	Solar saltern, Alicante, Spain	34.9 kDa; <60 °C; partially; narrow; ND; proton flux	Meseguer and Rodriguez-Valera (1985)
8	H5	Haloarchaeon Ma2.20	Solar saltern, Alicante, Spain	ND; ND; ND; narrow; ND; ND	Cheung et al. (1997)
9	H6/H7	*Haloferax gibbonsii* Ma2.39 or Alicante SPH7	Solar saltern, Alicante, Spain	32 kDa; <90 °C; no; narrow; Pronase; Na^+/H^+ Antiporter inhibitor	Torreblanca et al. (1989)
10	R1	*Halobacterium* strain GN101	Solar saltern, Mexico	3.8 kDa; <93 °C; no; broad; proteinase K; ND	Rdest and Sturm (1987)
11	S8	Strain S8a	Great salt Lake, Utah, USA	3.58 kDa; >100 °C; no; broad; proteinase K; ND	Price and Shand (2000)
12	KPS1	*Haloferax volcanii* KPSl	Kovalam saltern, Kanyakumari, Tamil Nadu, India	ND; <80 °C; ND; broad; proteinase K and trypsin; ND	Kavitha et al. (2011)

(continued)

Table 16.1 (continued)

S. No.	Halocin	Producer	Source	Properties (Molecular weight; thermostability; salt dependence; host range; protease sensitivity; mode of action)	References
13	Sech7a	*Haloferax mediterranei* Sech7a	Secovlje solar saltern, Slovenia	10.7 kDa; <80 °C; yes; narrow; ND; ND	Pasic et al. (2008)
14	SH10	*Natrinema* sp. BTSH10	Salt pan, Kanyakumari, Tamil Nadu, India	20 kDa; <50 °C; ND; ND; ND; ND	Karthikeyan et al. (2013)
15	HA1	*Haloferax larsenii* HA1	Pachpadra salt lake, Rajasthan, India	14 kDa, >100 °C; yes; narrow; proteinase K; membrane permeability	Kumar et al. (2016) and Kumar and Tiwari (2017a)
16	HA3	*Haloferax larsenii* NCIM5678 (HA3)	Pachpadra salt lake, Rajasthan, India	13 kDa, >100 °C; yes; narrow; proteinase K; membrane permeability	Kumar and Tiwari (2017b)

ND Not determined; kDa kilodalton

amino acid sequence and nucleotide sequence of the encoding gene have not been elucidated.

3. **Halocin H6:** Halocin H6 is produced from *Haloferax gibbonsii* Ma2.39 isolated from Spanish salt ponds in Alicante (Torreblanca et al. 1989). A halocin-overproducing mutant of strain Ma2.39 was named *Haloferax gibbonsii* Alicante SPH7. The halocin produced by strain SPH7 was designated halocin H7 (Torreblanca et al. 1989; Lequerica et al. 2006). The molecular weight of halocin H6 is 32 kDa. Similar to halocin H4, the production of halocin H6 reached maximum level at the transition from log to stationary phase (Torreblanca et al. 1989). Halocin H6 was heat-resistant and active up to 90 °C but activity reduced to 50% at 100 °C and completely lost at autoclaving temperature. Trypsin, even at a concentration of 5 mg/ml, does not denature the halocin activity (Torreblanca et al. 1989). Interestingly, halocin H6 is able to inhibit the sodium hydrogen exchanger (NHE) present in the cell membrane of haloarchaea and eukaryotic cell, but it was not tested against bacteria for NHE inhibition (Lequerica et al. 2006). The amino acid sequence of halocin H6 has not been determined (Torreblanca et al. 1989; Platas et al. 2002). Meseguer hypothesizes that mature halocin H6 would be in fact a 3 kDa peptide that is

released from a precursor protein, but the experimental data supporting this hypothesis are not available yet (Shand and Leyva 2007).

4. **Halocin SH10:** Halocin SH10 is produced by *Natrinema* sp. BTSH10 isolated from salt pan of Kanyakumari, Tamil Nadu, India. The molecular weight of halocin SH10 is 20 kDa. The halocin production reaches optimum level during the transition to the stationary phase of growth of producing strain incubated at 42 °C, pH 8.0, and 200 rpm in Zobell's medium containing 3 M NaCl (Karthikeyan et al. 2013). Halocin SH10 shows antimicrobial activity against halophilic isolates from saltern pond and particularly against *Halorubrum* sp. BTSH3. The proteinaceous nature of the antimicrobial activity of halocin SH10 has not been described (Karthikeyan et al. 2013; Besse et al. 2015).
5. **Halocin S8:** Halocin S8 is the first peptide halocin (microhalocin) produced by uncharacterized haloarchaeal strain S8a isolated from the Great Salt Lake in Utah, USA (Price and Shand 2000). The molecular weight of halocin S8 is 3.58 kDa. Similar to halocin H4 and H6, the production of halocin S8 reaches maximum at transition to the stationary phase of growth (Shand and Leyva 2007). Halocin S8 activity is thermostable up to 94 °C and sensitive to proteinase K but resistant to trypsin. It has salt-independent nature of activity (Price and Shand 2000). The desalted halocin S8 retains its activity and remains stable for months at 4 °C. Halocin S8 shows a narrow inhibitory spectrum. The CFS of producer strain inhibits the growth of *Halobacterium salinarum* NRC817, *Halobacterium* sp. strain GRB and *Haloferax gibbonsii* (Price and Shand 2000). Complete sequence of halocin S8 (36 amino acid residues) was revealed using Edman degradation method. Most of the residues are hydrophobic and consist of four cystine residues along the sequence which may form disulfide bridges. The information regarding its tertiary structure is unavailable. However, BLAST analysis of the halocin S8 sequence revealed no homology with other sequences available in the database (Shand and Leyva 2007; Besse et al. 2015).
6. **Halocin R1:** Halocin R1 is the second studied microhalocin produced by *Halobacterium salinarum* GN101, isolated from a solar saltern in Guerrero Negro, Mexico, by Barbara Javor (Ebert et al. 1986). The molecular weight of halocin R1 is 3.8 kDa. The production of halocin R1 reaches maximum during transition to the stationary phase of growth of the producing strain. Halocin R1 activity is found to be resistant up to 60 °C for 24 h but lost after treatment at 93 °C for 5 min. The halocin R1 activity is resistant to desalting and to various proteolytic enzymes such as papain, trypsin, and thermolysin, but it is sensitive to proteinase K, pronase, and elastase (Besse et al. 2015). The complete sequence of halocin R1 was determined using Edman degradation. It consists of 38 amino acids that are 63% identical and 71% similar to halocin S8 (O'Connor and Shand 2002; Price and Shand 2000). The halocin R1 is reported to be archaeostatic as suggested by Rdest and Sturm (1987). The halocin R1 producing strain GN101 is studied for complete sequence of megaplasmid 2 (283 kb) and is available in GenBank database under accession number EU080936. It carries *hal*S8 gene that suggests for the strong relationship between halocins S8 and R1 and might be encoded by same gene or multiple

deriving copies of the halocin S8 gene. Both halocin S8 and R1 are able to inhibit the growth of *Sulfolobus* species, whereas halocin R1 is also able to inhibit *Methanosarcina thermophila* (Haseltine et al. 2001; Besse et al. 2015).

7. **Halocin C8:** Halocin C8 is the largest member of the microhalocin family produced by haloarchaeon *Natrinema* sp. AS7092 (formerly *Halobacterium* strain AS7092), isolated from the Great Chaidan Salt Lake, China (Li et al. 2003; Sun et al. 2005). The molecular weight of halocin C8 is 7.44 kDa. It has been reported that several strains of *Natrinema* are able to produce halocin C8. The gene responsible for halocin production is reported in five other strains of *Natrinema* species isolated from the Ichekaben salterns in Algeria (Imadalou-Idres et al. 2013). The halocin production began at the transition from the exponential to the stationary phase of growth, and activity remains stable throughout the stationary phase (Sun et al. 2005). The halocin C8 is thermostable up to 100 °C for 1 h and salt-independent in nature. The desalted halocin may be stored for more than 1 year at 20 °C without losing the activity. The activity remains unaffected after treatment with various organic solvents such as methanol, ethanol, and acetonitrile. It is sensitive to proteinase K and papain but resistant to trypsin. These properties make halocin C8 a quite stable peptide and robust in nature (Li et al. 2003). Halocin C8 is not able to inhibit bacteria, whereas it shows wider inhibitory spectrum against different strains of haloarchaea. It inhibited the growth of 16 out of the 21 strains of haloarchaea including three alkaliphilic haloarchaea such as *Natronobacterium gregoryi*, *Natronobacterium magadii*, and *Natronomonas pharaonis* (Li et al. 2003). The halocin C8 sequence is cysteine-rich and contains ten cysteine residues which may form disulfide bridges responsible for higher stability of halocin C8 activity in different conditions (Li et al. 2003; Sun et al. 2005).
8. **Halocin A4:** Halocin A4 is produced by an uncharacterized haloarchaeon strain TuA4 isolated from Tunisian saltern (Shand and Leyva 2008). Previously, halocin A4 was also designated halocin U1. The molecular weight of halocin A4 is 7.4 kDa. The halocin A4 activity is salt-independent and thermostable. It is resistant to boiling temperature up to a week (Shand and Leyva 2007; Shand and Leyva 2008). Like halocin C8, it has same molecular mass and similar stability pattern. It shows broad inhibitory spectrum against other haloarchaea and is able to inhibit *Sulfolobus solfataricus* (crenarchaeal hyperthermophile) similar to halocin S8 and R1 (Haseltine et al. 2001). N-terminal sequencing of halocin A4 revealed 75% identity to halocin C8, thus suggesting it is a variant of halocin C8 (Shand and Leyva 2008).
9. **Halocin Sech7a:** Halocin Sech7a is produced by *Haloferax mediterranei* Sech7a, isolated from Adriatic solar saltern in Slovenia. The molecular weight of halocin Sech7a is 10.7 kDa. Halocin production starts when cells enter into the exponential phase of growth (30 h) and reaches maximum level at the beginning of the stationary phase (40 h) grown at 45 °C and pH 7.0–7.5 and 20% NaCl (Pasic et al. 2008). It is quite stable in a wide range of pH 2.0–10.0 and thermostable up to 80 °C. The activity remained unaffected at $\geq$6% NaCl, but further desalting reduces the activity. Its activity can be restored up to ~40%

of initial halocin activity by dialysis against the initial saline conditions (Pasic et al. 2008; Besse et al. 2015).

10. **Halocin KPS1:** Halocin KPS1 is produced by *Haloferax volcanii* KPSl, isolated from Kovalam saltern, Kanyakumari, India. The growth and halocin production have been monitored at 25% NaCl, 40 °C, and pH 7.0 (Kavitha et al. 2011). The production of halocin KPS1 starts during mid-log phase at 48 h, reaches maximum (120 AU/mL) at the stationary phase of growth at 72 h, and persists up to 120 h; thereafter, activity was declined (40 AU/ml) at 144 h (Kavitha et al. 2011). Halocin KPS1 activity is thermolabile and reduced at >80 °C and stable over a wide range of pH 3.0–9.0. The activity disappears after treatment with proteolytic enzymes such as proteinase K and trypsin indicating the proteinaceous nature of the inhibitory compound. Halocin KPS1 shows broad inhibitory spectrum and is active against Gram-positive and Gram-negative bacteria such as *Bacillus subtilis* MTCC1134, *Streptococcus mutans* MTCC896, *Staphylococcus aureus* MTCC916, *Escherichia coli* MTCC1671, and *Pseudomonas aeruginosa* MTCC6538, respectively. It shows strong antimicrobial activity against *Halobacterium sodomens* S2 (Kavitha et al. 2011; Besse et al. 2015).
11. **Halocin HA1 and HA3:** Halocin HA1 and HA3 were produced by *Haloferax larsenii* HA1 and HA3 isolated from Pachpadra salt lake in Rajasthan, India. Halocin HA1 and HA3 were purified using ultrafiltration, anion-exchange chromatography (AEC), gel-filtration chromatography (GFC), and reverse-phase high-performance liquid chromatography (RP-HPLC). It was observed that purified halocin HA1 was stable up to 80 °C and lost 50% activity at 100 and 121 °C, whereas halocin HA3 activity was reduced up to 88% at 100 °C and 94% at 121 °C. Halocin HA1 and HA3 were active at pH 4.0–12.0 and pH 2.0–10.0, respectively, suggesting for their stability under acidic to basic pH range. Purified halocin HA1 and HA3 were unaffected with methanol, ethanol, isopropanol, Tween-80 and Triton X-100 but showed complete loss of activity after treatment with trypsin and proteinase K. Both halocins showed the salt-dependent nature of antimicrobial activity and required minimum 10% NaCl for activity. The molecular weights of purified halocin HA1 and HA3 were found to be ~14 and ~13 kDa, respectively, using tricine SDS-PAGE. MALDI-TOF MS/MS analysis of halocin HA1 and HA3 showed no homology with known halocins in NCBI database. The N-terminal sequences of halocin HA1 and HA3 were found to be MIDREILEVN and MNLGIILETN, respectively. Blastp (NCBI) analysis of N-terminal sequence of halocin HA1 showed no significant homology with known proteins, whereas halocin HA3 showed 100% identity with N-terminal of hypothetical protein of *Halogeometricum pallidum* (Kumar et al. 2016; Kumar and Tiwari 2017a, b).

Sulfolobicins These are the archaeocins produced from several members of genus *Sulfolobus* belonging to phylum Crenarchaeota. The members of genus *Sulfolobus* grow in entirely different environments such as high temperature and acidic conditions. Sulfolobicins are entirely different from halocins and their activity is due to cells and not the supernatant (Besse et al. 2015). Sulfolobicin is the

proteinaceous antimicrobial agent, first characterized from *Sulfolobus islandicus*. It is not the secretory proteins but remains bound to the cell membranes or cell-derived S-layer-coated membrane vesicles (Prangishvili et al. 2000). The host range of sulfolobicin was found to be restricted to other members of the *Sulfolobales*. The sulfolobicin from *Sulfolobus islandicus* inhibited *Sulfolobus solfataricus* P1, *Sulfolobus shibatae* B12, and six nonproducing strains of *Sulfolobus islandicus* but did not inhibit *Sulfolobus acidocaldarius* DSM639. The purified sulfolobicin from strain HEN2/2 did not inhibit *Halobacterium salinarum* R1 or *Escherichia coli*. Sulfolobicin was purified from the late stationary phase culture using sonication high-speed centrifugation and separated with Triton X-100. The molecular weight of sulfolobicin was 20 kDa tested on SDS-PAGE (Prangishvili et al. 2000; Besse et al. 2015). Its activity remained stable up to 6 months. Enzymatic treatment with α-amylase, α- and β-glucosidases, phospholipase C, and lipoprotein lipase did not affect the sulfolobicin activity, whereas treatment with proteolytic enzymes such as pronase E, proteinase K, and trypsin showed complete loss of activity (Shand and Leyva 2007). The study of genes responsible for sulfolobicin synthesis and resistance would be useful to select as genetic markers that are still scarce in *Sulfolobus* (Ellen et al. 2011).

16.4 Applications

Haloarchaea produce novel biomolecules for various industrial applications. The commercial importance of haloarchaea and their products has been recorded since ancient times. Due to red-purple color of most of the members, they absorb solar radiation and speed up the evaporation process in hypersaline environment leading to the increased production of salts. The biomolecules produced by haloarchaea play a significant role for various applications in fermented food products, cosmetics, preservatives, biosensor, etc. (DasSarma et al. 2010). A large number of applications in various sectors like chemical, environmental, biofuel, pharmaceutical, healthcare, and food industries are likely to be available in the future. The applications of haloarchaea and their various biomolecules are described as below:

1. **Retinal proteins**: It is one of the most recognized products derived from halophilic Archaea. Almost 80 US patents pertaining to bacteriorhodopsin from *Halobacterium* have been reported. It converts light to chemical energy, first discovered in the haloarchaea, using light to establish a transmembrane H^+ gradient (DasSarma et al. 2010). Bacteriorhodopsin from *Halobacterium* species is being marketed for light sensors, nonlinear optics, and optical data processing. It can be used for providing sights to the robots used in the industry. It is also being considered for use as an erasable photochromic film. Bacteriorhodopsin films also have the potential to be used as biochips which could pass electrical signals, therefore replacing the integrated circuits in modern computers (Ratnakar 2013). The cell membrane of haloarchaea contains ether-

linked lipids and often has different retinal proteins, mainly bacteriorhodopsin, halorhodopsin, and sensory rhodopsins (Ma et al. 2010).

2. **Osmolytes:** Haloarchaea accumulate small organic compounds inside the cells known as osmolytes to maintain the osmotic balance. A great variety of osmotic solutes have been identified such as different amino acid derivatives (e.g., glycine betaine and ectoine) or sugars and polyols (e.g., sucrose, trehalose, and glycerol) which do not disrupt metabolic processes and have no net charge at physiological pH (DasSarma et al. 2010). These osmotic solutes may be accumulated via either de novo biosynthesis or direct uptake from the medium. Compatible solutes can act as stabilizers for biological structures and allow the cells to adapt not only to salts but also to heat, desiccation, and cold. It also acts as protectant for DNA (DasSarma and DasSarma 2012; Yin et al. 2015). Few members of haloarchaea produce most common osmotic solute such as ectoine that can protect many unstable enzymes and nucleic acids against denaturation from high salt, temperature, and desiccation, therefore increasing the shelf life and activity of enzyme preparations (Britton et al. 2006; Ratnakar 2013). Ectoine inhibits conversion of trypsinogen to trypsin and also stabilizes the activity of trypsin and chymotrypsin and also called as "molecular chaperones". They have been used as moisturizers in cosmetics for the care of aged, dry or irritated skin. Ectoine and its derivatives have been patented as moisturizers in cosmetics and as stabilizers in polymerase chain reactions (Oren 2010). *Halomonas boliviensis* is being explored for its use in the production of ectoine. Glycine betaine, a common osmolyte, has been proposed as a feed additive (Guzman et al. 2009). *Halomonas boliviensis* is being explored for its use in the production of ectoine in batch-fed cultures. Its biosynthetic pathway has been characterized and genetically engineered to express *Ectothiorhodospira halochloris* genes in *Escherichia coli*, resulting in the accumulation of betaine with improved salt tolerance (DasSarma et al. 2010).
3. **Fermented foods:** Haloarchaea have been widely used in the preparation of fermented food products (Roh and Bae 2009). Kimchi, a traditional Korean food based on fermented vegetables, showed the presence of a highly diverse community of halophilic Archaea, lactic acid bacteria, and yeasts (Lee 2013). Few members such as Halobacteria, Halococci, and Natronococci have been isolated from various fermented foods and sauces including kimchi (Hiraga et al. 2005; Namwong et al. 2006). Large-scale industrial productions of soy sauce and fish sauce exploit the use of the degradative powers of haloarchaea. Both hydrolases and isomerases from halophilic Archaea may find increased application in the food industry. Various traditionally salt-fermented foods are especially popular in the Far East (DasSarma et al. 2010). Examples are "jeotgal", Korean fermented seafood, and "fugunoko nukazuke," Japanese fermented food prepared from salted puffer fish ovaries in rice bran. Due to high salt concentration, the haloarchaeal growth and their protease production probably take part in the fermentation process that contributes aroma to the sauce. Fermentations that involve lower salt concentrations generally give rise to the development of moderately halophilic Achaea such as *Halobacillus* and *Halomonas* (Hong 1986; Vsevolodov and Dyukova 1994).

4. **Carotenoids:** Natural pigments in the cell membranes of microbes provide attractive colors and play basic biological roles in their development (Delgado-Vargas et al. 2000). Carotenoids are one of the natural pigments and their biotechnological applications and beneficial effects on human health have been reported (Vílchez et al. 2011; Fiedor and Burda 2014; Zhang et al. 2014). These compounds are the second most abundant naturally occurring pigments in nature ranging from colorless to yellow, orange, and red (Yatsunami et al. 2014; Nisar et al. 2015). Carotenoids, hydrophobic compounds, are of two types: carotenes or carotenoid hydrocarbons, e.g., lycopene and β-carotene that are composed of carbon and hydrogen only, and xanthophylls or oxygenated carotenoids, e.g., lutein, canthaxanthin, zeaxanthin, violaxanthin, capsorubin, and astaxanthin that may contain epoxy, carbonyl, hydroxyl, methoxy, or carboxylic acid as functional groups (Rivera and Canela-Garayoa 2012; Fassett and Coombes 2012). The biosynthesis and fate of carotenes and xanthophylls and their role have been well described in plants (Nisar et al. 2015) and mammals (Palczewski et al. 2014). Animals are not able to synthesize carotenoids de novo, and consequently, they acquire through diet. In most of the organisms, carotenoids show powerful antioxidant properties, which directly emerge from their molecular structure (Rodrigo-Baños et al. 2015).

   The carotenoids of haloarchaea are scarce as compared to other organisms (Naziri et al. 2014). Several members that belong to the families *Haloferacaceae* and *Halobacteriaceae* are able to synthesize C_{50} carotenoid pigment such as bacterioruberin. Carotenoids including phytoene, lycopene, and β-carotene are also produced by *Haloferax volcanii* but at lower concentration. Usually, these carotenoids are in the cell membranes, and they provide color to the colonies. Bacterioruberin and its derivatives provide protection against UV radiations. The synthesis of carotenoids is induced by low oxygen tension, high light intensity and high osmotic stress (Kelly and Jensen 1967; Kushwaha et al. 1975; El-Sayed et al. 2002; Rodrigo-Baños et al. 2015). The production of carotenoids can be increased using genetic engineering or modifications of different parameters like growth, pH, temperature, etc. Haloarchaea accumulate carotenoids at higher salinity that is being produced commercially in large-scale outdoor facilities. β-Carotene extracted from haloarchaea has been used in vegetable oil, food coloring agent in margarine, baked goods, and some prepared foods. β-Carotene is pro-vitamin A and is an excellent source of this vitamin since it is nontoxic even in high doses (Schiraldi et al. 2002). There are few patents in which the potential uses of haloarchaeal carotenoids in biomedicine and biotechnology have been tested (Rodrigo-Baños et al. 2015) that may be helpful to explore further biotechnological usage of haloarchaeal carotenoids at large scale.

5. **Halophilic enzymes:** Most of the enzymes isolated from haloarchaea have been reported to be stable at higher salinity because of the presence of higher acidic amino acid residues, low hydrophobic residues, and lower frequency of lysine residue. This composition makes the protein's surface acidic with a decrease in hydrophobic patches (Britton et al. 2006; DasSarma and DasSarma 2015). They contain predominance of negatively charged residues on the solvent-exposed

surfaces of the protein, and these negative charges attract water molecules and make the proteins hydrated; therefore, they do not precipitate (Balasubramanian et al. 2002). Most of the halophilic enzymes from haloarchaea are generally active with low water activity. These enzymes can be used in making biocatalysts in the presence of organic solvents (Ratnakar 2013). Halophilic enzymes also demonstrate thermophilic character and remain stable in a broad range of temperatures (Eichler 2001; Munawar and Engel 2013) that make them potential for biotechnological applications. Few enzymes from haloarchaea have been described as below.

(i). ***Proteases*****:** Proteolytic enzymes are used to produce pharmaceutical products, foods, detergents, leather, silk, agrochemical products, etc. In terms of production, proteases represent the heart of the global market for enzymes (Adrio and Demain 2014). Haloarchaeal proteases show optimum activity at high salt concentration, although some of them can be stable and active at lower concentrations. The extracellular endopeptidase from *Halobacterium halobium* hydrolyzes polypeptides and oligopeptides with specificity for hydrophobic amino acids, especially proline (Capiralla et al. 2002). This endopeptidase could be an important tool to be used in the food processing industry as well as in biomedical applications to produce peptides (Capiralla et al. 2002). Kim and Dordick studied the stability of a protease from *Halobacterium halobium* in different aqueous/organic solvent mixtures, and they observed that it correlates strongly with the salting-out ability of the solvent. The possibility of using these enzymes in hydrophobic medium increases the potential biotechnological applications (Kim and Dordick 1997). Proteases from haloarchaea are widely used in the detergent and food industries. They are also used in the leather industry and in the preparation of soy products and aspartame. Proteolytic activity of proteases from various members of haloarchaea such as *Halobacterium* spp., *Haloferax mediterranei*, *Natrialba asiatica*, *Natrialba magadii*, *Natronococcus occultus*, and *Natronomonas pharaonic*, has been characterized along with their potential industrial applications (DasSarma et al. 2010).

(ii). ***Glycosyl hydrolases*****:** Glycosyl hydrolases are enzymes capable of hydrolyzing glucosidic bonds between carbohydrates. They are classified into 108 families based on amino acid similarities. Among them, glycosyl hydrolase family 13 is of special interest that contains various starch-modifying enzymes including α-amylases, α-1,6-glucosidases, pullulanases, branching enzymes, maltogenic amylases, neopullulanases, and cyclodextrinases (Labes et al. 2008). Amylases from halophilic Archaea including moderately halophilic strains (e.g., *Halomonas meridiana*) to extremely halophilic strains (e.g., *Haloarcula hispanica* and *Natronococcus amylolyticus*) have been characterized (DasSarma et al. 2010). Most of them retain their activity at high temperatures. For example, the haloarchaeon *Haloferax mediterranei* secretes an α-amylase showing optimum temperature between 50 and 60 °C, but it retains 65%

of the maximum activity at 80 °C (Pérez-Pomares et al. 2003). Cyclodextrins are interesting molecules produced from starch by enzymatic breakdown. The amylase of *Haloarcula* sp. works optimally at 4.3 M salt and 50 °C, but the enzyme does not lose its activity at low salt concentrations. Even in the absence of NaCl, it maintains more than 30% activity. The enzyme is also stable in benzene, chloroform, and toluene demonstrating its potential as a good candidate for industrial applications (Hutcheon et al. 2005). Glycosyl hydrolases have been used in degradation of a wide variety of products such as cellulose, agar, agarose, lactose, and amylose. Xylanase and xylosidase from *Halorhabdus utahensis* have been used for the complete degradation of xylan, a main component of hemicellulose. Xylanases have been also used in the manufacturing of coffee (Woodward and Wiseman 1984), livestock feeds (Veldman and Vahl 1994), and flour (Hilhorst et al. 1999). Agarase, glycosyl hydrolase, hydrolyzes the agar and, therefore, is used in the laboratory and industrial setting for extracting DNA and other embedded molecules from agarose as well as extracting bioactive or medicinal compounds from algae and seaweed (DasSarma et al. 2010).

(iii). ***Amylases*****:** Amylases are produced from many strains of haloarchaea including *Halomonas meridiana* (Coronado et al. 2000), *Haloarcula hispanica* (Hutcheon et al. 2005), and *Natronococcus amylolyticus* (Ross et al. 2002), and these have been characterized and used industrially for the production of corn syrup with high level of fructose. All amylases are glycoside hydrolases and act on α-1,4-glycosidic bonds. Starch is the main source of carbohydrates consisting of amylose and amylopectin (Parka and Son 2007). Amylases, an industrially important enzyme, have wider applications such as in starch processing, alcohol production, food, textile, pharmaceutical, and detergent industries. This enzyme hydrolyzes the starch and converts it into smaller units. In baking industry, α-amylase can be used to improve the taste and aroma of the bakery products. In alcohol industry, starch is converted to fermentable sugars using α-amylase. Ethyl alcohol production is carried out by fermentation of starch-rich substrates like potatoes and certain grains. In detergent industry, stabilizing the bleaching effect is one of the major uses of the amylase enzyme (Thombre et al. 2016). For the cattle feed industry, the addition of α-amylase improves the body weight gain and feed conversion ratio. α-Amylase is also known to improve the paper quality and its stiffness and also helps in sizing and coating of the paper. It also protects the paper from mechanical injury (Bozic et al. 2011).

(iv). ***β-Galactosidases*****:** It is a glycoside hydrolase enzyme and hydrolyzes β-galactosides into monosaccharides through breakdown of glycosidic bond. In a study of β-galactosidase from *Halorubrum lacusprofundi*, a cold-adapted haloarchaeon, the enzyme was found to be active over a broad temperature range (−5 to 70 °C) under optimal conditions (4–5 M NaCl or KCl) (Karan et al. 2013). It was stable and active in aqueous

solutions of alcohols, including methanol and ethanol. The basis for these characteristics was explained by bioinformatic analysis like homology modeling and predicted that certain amino acid residues at the enzyme surface and interior are critical for its halophilic character. Additional mutagenic studies of this enzyme are likely to lead to valuable insights into adaptation to both temperature and salt extremes in the future. It has been also used for the removal of lactose from dairy products (DasSarma et al. 2010; DasSarma and DasSarma 2015).

(v). ***Cellulases and chitinases*****:** Halophilic cellulases have recently generated interest by their application in biofuel production. Plant biomass acts as starting material and consists mainly of cellulose, hemicellulose, and lignin. The latter one is highly resistant to biodegradation processes and involves the use of harsh pretreatments (high temperatures and extreme pH conditions) to degrade. The ionic liquids (ILs) can also efficiently solubilize cellulose, hemicellulose, and lignin under moderate temperatures. Enzymes from haloarchaea are resistant to these harsh conditions (Hatori et al. 2006; Zhang et al. 2011). Zhang et al. have identified and characterized a halophilic cellulase (Hu-CBH1), a heat-tolerant haloalkaliphilic enzyme isolated from the halophilic archaeon *Halorhabdus utahensis*. It is active in salt concentrations up to 5 M NaCl, pH 11.5, and high levels of ILs (Zhang et al. 2011).

Chitinases (glycosyl hydrolases) breakdown β-1,4-glycosidic bonds present in chitin. Besides cellulose and starch, it is one of the most abundant polysaccharides in nature. The main natural chitin sources are the shells of crustaceans, insect exoskeletons, and fungal cell walls. A chitinolytic extremozyme from the halophilic archaeon *Halobacterium salinarum* showed the highest activity in the presence of 1.5 M NaCl, even retaining 20% of its activity in the absence of salt. Hou et al. identified the genes and described the enzymatic breakdown process of chitin in *Haloferax mediterranei* (Hou et al. 2014). The chitinolytic enzymes have wider applications including preparation of chitooligosaccharides and *N*-acetylglucosamines in the pharmaceutical industries (Dahiya et al. 2006).

(vi). ***Esterases and lipases*****:** These enzymes hydrolyze ester bonds between a fatty acid moiety and an esterified conjugate like glycerol or phosphate. Lipases preferentially hydrolyze triglycerides composed of long-chain fatty acids, while esterases usually hydrolyze water-soluble esters, including short-chain fatty acid triglycerides. These enzymes are being used in various sectors like pharmaceutical, cosmetic, leather, and paper industries; textile, biopolymer, and biodiesel production; pretreatment of lipid-rich wastewaters; food modification; and detergent formulation (Anobom et al. 2014). These processes often require aggressive reaction conditions like high temperatures to sustain biocatalysis, organic solvents as part of the reaction mixtures, or the presence of high salt concentration. Thus, the enzymes such as halophilic lipases and thioesterases produced

by haloarchaea have potential biotechnological applications and required more attention in such field for large-scale applications (Schreck and Grunden 2014). Bhatnagar et al. isolated *Natronococcus* from a salt lake in Algerian Sahara. It secretes lipase that was optimally active at 4 M NaCl, pH 7, and 50 °C (Boutaiba et al. 2006). An esterase from *Haloarcula marismortui* has recently been purified and characterized for various possible applications (Muller-Santos et al. 2009). Moreover, Muller-Santos et al. cloned and overexpressed the *lipC* gene from *Haloarcula marismortui* (Muller-Santos et al. 2009). The recombinant protein was purified and characterized for its biochemical properties. The lipases from haloarchaea are used in biofuel production. Therefore, a lipase from a haloarchaeal strain G41 was purified to homogeneity and characterized (Li and Yu 2014). The enzyme displayed high stability and activity in the presence of hydrophobic organic solvents and showed preference toward long-chain substrates, which makes the enzyme suitable for biofuel production. The free and immobilized lipase from strain G41 was applied for biodiesel production, and 80.5 and 89.2% of yields were achieved, respectively (Li and Yu 2014). This suggested the feasibility of using lipases for the production of biodiesel. Esterases and lipases are also widely used as biocatalysts and useful ingredients in laundry detergents for the removal of oil/grease stains (DasSarma et al. 2010).

(vii). ***Restriction enzymes*:** Restriction endonucleases such as HcuI, HhlI, and HsaI have been isolated from *Halobacterium cutirubrum*, *Halobacterium halobium*, and *Halobacterium salinarum*, respectively. These enzymes have been produced on a commercial scale and play a major role in molecular biology field applicable to industrial level. Endonuclease (HacI) has been isolated from *Halococcus acetoinfaciens*, and the process for its production has been patented. A patent for production of a novel restriction enzyme of unusual specificity from a *Halococcus* sp. has been filed (Obayashi et al. 1988; DasSarma et al. 2010).

6. **Polyhydroxyalkanoates (PHAs):** These are polyesters composed of hydroxy fatty acids, synthesized and stored as insoluble inclusions in the cytoplasm (Poli et al. 2011). They serve as intracellular storage material of carbon source and energy. PHAs are produced in the stationary phase of growth, when the medium is deficient in some essential nutrients but a carbon source is available in excess. Haloarchaea uses the degradation products of PHAs or depolymerized form for their growth (Mozejko-Ciesielska and Kiewisz 2016). PHAs are classified into two distinct groups on the basis of number of carbon atoms in the monomers (Olivera et al. 2010; Mozejko-Ciesielska and Kiewisz 2016): scl-PHAs (short-chain length PHAs) and mcl-PHAs (medium chain length PHAs). scl-PHAs contain monomers of 3–5 carbon atoms, whereas mcl-PHAs contain monomers of 6–14 carbon atoms. On the other hand, PHAs can be classified on the basis of type of monomers involved (Olivera et al. 2010; Meng et al. 2014): homopolymers, made of identical monomers, include PHB (poly-3-

hydroxybutyrate), P3HP (poly-3-hydroxypropionate), P4HB (poly-4-hydroxybutyrate), PTE (polythioester), PLA (polylactic acid), and PHV (polyhydroxyvalerate), whereas copolymers (also called heteropolymers) derived from more than one species of monomer like PHBV (polyhydroxybutyrate-valerate) (Mozejko-Ciesielska and Kiewisz 2016). PHAs are synthesized by four natural pathways with involvement of PHA synthases (Rehm 2003; Poli et al. 2011; Meng et al. 2014). Based on subunit compositions and their substrate, these proteins are divided into four classes in case of domain Bacteria (Rehm 2003; Poli et al. 2011; Mozejko-Ciesielska and Kiewisz 2016). PHA synthases of Classes I and II consist of only one type of subunit (PhaC) with molecular masses between 61 and 73 kDa. The difference between Classes I and II lies in the substrate specificity. The PHA synthases of class III consist of two subunits, PhaC and PhaE, both with similar molecular weight (~40 kDa). Class IV PHA synthases are like class III but the PhaE subunit is replaced by PhaR (with molecular mass ~20 kDa) (Rehm 2003; McCool and Cannon 2001). PHAs have been extensively studied in the Bacteria domain. However, in the Archaea domain, knowledge is more limited, although haloarchaea seem to be good models to produce these biopolymers.

PHAs are reported in various members of genera *Haloferax, Halococcus, Halobacterium, Haloarcula, Haloterrigena, Haloquadratum, Halorubrum, Natronobacterium, Natronococcus*, and *Natrialba*. Haloarchaea such as *Haloarcula marismortui* and *Haloferax mediterranei* are capable of producing large amounts of PHA using glucose and/or starch as substrates (Oren 2010; Poli et al. 2011). The archaeal PHA synthases are composed of two subunits, PhaE and PhaC, that are homologous to the class III PHA synthases from bacteria with only two differences: First, they present a longer C-terminal extension in the PhaC subunit. Second, the PhaE subunit lacks the hydrophobic and amphiphilic amino acids for granule association, and it is much smaller than its bacterial counterpart (Han et al. 2007; Lu et al. 2008). The advantages of using haloarchaea to produce PHAs are numerous: They require simple growth medium; high salt concentrations in their growth media prevent contamination from other organisms; therefore, the requirements for sterile conditions can be reduced (Don et al. 2006; Alsafadi and Al-Mashaqbeh 2017). Moreover, the biopolymers obtained can be easily recovered by osmotic shock of cells using media with low salinity or even distilled water (Alsafadi and Al-Mashaqbeh 2017), so it is not necessary to use any solvents to extract them. The two most studied genera are *Haloarcula* and *Haloferax* reported for higher PHA producers. PHB was extracted from *Haloarcula* sp. IRU1, as biopolymer with a yield of 63% (w/w) of cell dry weight (CDW), and glucose was used as carbon source (Taran and Amirkhani 2010). The yield of 21 and 2.4% (w/w) CDW was reported in the case of *Haloarcula marismortui* and *Haloarcula hispanica*, respectively. *Haloferax mediterranei* is probably the best-studied strain in terms of producing PHAs (Don et al. 2006; Han et al. 2007). Besides the useful properties of haloarchaea to produce PHAs, *Haloferax mediterranei* is having the characteristics of high growth rate and ability to produce copolymer PHBHV

(Alsafadi and Al-Mashaqbeh 2017). PHBHV is a copolymer form of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3 HV). It presents improved properties compared to the homopolymer PHB, which is a brittle plastic that considerably limits its application (Don et al. 2006).

Because of various industrial applications of PHAs as biodegradable and biocompatible polymers, they receive considerable attention nowadays (Oren 2010). PHAs can be applied in various fields. In the packaging industry, they can compete with nondegradable polymers in the production of bottles and containers (Quillaguamán et al. 2010; Bugnicourt et al. 2014). Due to PHB's compatibility with mammalian tissues and blood, it can be used in biomedicine sector as osteosynthetic products, sutures, and wound dressing materials (Don et al. 2006; Bugnicourt et al. 2014). Numerous companies develop and commercialize different types of PHAs such as Biomer (Germany), PHB Industrial (Brazil), Bio-on (Italy), or Telles LLC (USA) and sell these biopolymers under the commercial name Metabolix. PHAs produced by various members of haloarchaea as mode of carbon storage are used for manufacturing bioplastics. In spite of various advantages, their production on a large scale is still complicated because of high production cost (7–10 euros/kg) (Bugnicourt et al. 2014), and PHA processing is more difficult than petrochemical plastics due to their slow crystallization processes (Laycock et al. 2013) as well as PHAs do not have consistent structures and properties compared to conventional plastics (Wang et al. 2014). Till date, the currently used PHAs cannot replace plastics derived from the petrochemical industry. Therefore, future research is required to focus on its better production, cost reduction, innovation in the use of waste carbon sources, strain improvements and short downstream process with better recovery. Due to low nutritional requirements, haloarchaea may be used as models to achieve these objectives. In contrast to many bacterial species, haloarchaea can be used to obtain biopolymers easily.

7. **Biodegradation of hydrocarbons:** Different members of haloarchaea are involved in biodegradation of hydrocarbons such as benzene, toluene, ethyl benzene, and xylene present in hypersaline environments (Berlendis et al. 2009). *Haloferax* and *Halorubrum* spp. have been used for the biotreatment of highly saline industrial effluents. *Halomonas* sp. HTB24 isolated from hypersaline olive oil wastewater has been reported to convert the tyrosol into antioxidant hydroxytyrosol (HT) and 3,4-dihydroxyphenylacetic acid (DHPA) that further degraded to succinate and pyruvate (Liebgott et al. 2007). *Halobacterium* sp. NRC-1 has been used as model haloarchaeon that produces unique gas-filled organelles called gas vesicles. It allows these microbes to float to overcome the oxygen deficiency in hypersaline environment. The genetic basis for gas vesicles production has been characterized and designed a vector that is involved in biodegradation as well as floating property after transformation (DasSarma and Arora 1997). The genetically engineered microbes were transformed with dual characteristics. Production of biosurfactants has been reported from various members of haloarchaea having potential

biotechnological applications and that are able to treat hydrocarbon pollution in marine aquaculture (Banat et al. 2000; Kebbouche-Gana et al. 2009).

8. **Wastewater treatment:** Textile industry generates large volumes of polluted wastewater containing phenol, azo dyes, and various toxic products and creates salinity up to the level of 15–20% salt. Moderately halophilic Archaea such as *Halomonas* species have been reported from these polluted wastewaters and are able to utilize phenol as a carbon and energy source as well as decolorize the azo dyes in wastewater (Guo et al. 2008). The wastewater treatments or breakdown of sewage influent are generally performed by microorganisms which are able to live in the sludge of treatment plants and holding tanks. They obtain nutrients by degrading the solids to various compounds. Some wastewater treatment systems are efficient and economically important. The establishment of optimal conditions such as nutrients, pH, temperature, and oxygen availability is an important parameter for optimal growth of microbes to efficiently treat the sewage influent. The industrial wastewater treatments enriched with ammonia, phenol, and high salinity constrain the microbial populations closely related to *Methanobrevibacter smithii* (methanogen) found in human intestines (Bonete et al. 2015). The manufacturing of pesticides, herbicides, explosives, etc. usually generates effluents containing complex mixtures of salts and nitrate or nitrite. Also, the increase of salinity in soils and waters gives advantage to some species like *Haloferax mediterranei*. For example, *Haloferax mediterranei* is resistant to very high nitrate (up to 2 M) and nitrite (up to 50 mM) concentrations, which are the highest compared to other prokaryotic microorganisms. Therefore, it can be applied for bioremediation purpose in sewage plants where high salts, nitrate, and nitrite concentrations are detected. It was able to eliminate 60% of the nitrate and 75% of nitrite (Najera-Fernandez et al. 2012).
9. **Denitrification:** Many haloarchaea species have reductive pathways of nitrate assimilation and nitrogen fixation as well as dissimilatory pathway like nitrate respiration and denitrification. Nitrogen metabolism is less studied in haloarchaea than in bacteria. However, in *Haloferax mediterranei*, some of these pathways are well known and considered it as a good candidate for bioremediation process. Haloarchaea can reduce nitrate through nitrate and nitrite reductase enzymes during assimilatory or respiratory pathways. These enzymes are found in a variety of halophilic and hyperthermophilic Archaea. Several members of haloarchaea belonging to the genera of *Haloferax* and *Haloarcula* help in denitrification. The enzymes mainly glutamine synthetase, glutamate synthase, and glutamate dehydrogenase are responsible for ammonium assimilation in haloarchaea such as *Haloferax mediterranei* (Bonete et al. 2015). *Haloferax mediterranei* acts as denitrifier and is able to utilize NO_3 and NO_2 as inorganic nitrogen sources and therefore used in bioremediation of nitrate and nitrite concentrations in hypersaline wastewater (Bonete et al. 2008). The toxic pollutants available in hypersaline wastewater may cause disease like blue baby syndrome. However, the haloarchaea help in preventing

such type of disease and reduce the toxic level of wastewater (DasSarma et al. 2010).

10. **Heavy metal cleanup:** Due to readily available heavy metals in hypersaline environments, many members of haloarchaea developed tolerance to these metals. *Halobacterium* sp. NRC-1 was reported to be resistant to arsenic due to the presence of plasmid-encoding arsenite and antimonite extrusion system base gene that help in ion arsenic cleanup (Wang et al. 2004). Similarly, other members of haloarchaea are able to detoxify lead and cadmium (Amoozegar et al. 2007).
11. **Halocins:** Halocins are the natural antimicrobial compounds which can control the growth of haloarchaea in salted food products; therefore, it may have potential application in food industry as preservative agents (Charlesworth and Burns 2016). Halocins such as HA1, HA3 (Kumar and Tiwari 2017a, b), Sech7a (Pasic et al. 2008), H4 (Meseguer and Rodriguez-valera 1985), H6 (Torreblanca et al. 1989), C8 (Li et al. 2003), and SH10 (Karthikeyan et al. 2013) have been reported which suggest that these halocins killed the indicator organisms by cell swelling followed by cell lysis (O'Connor and Shand 2002; Sun et al. 2005; Pasic et al. 2008; Karthikeyan et al. 2013). The halocin-treated sensitive cells demonstrated change in internal pH, membrane potential, proton motive force, and sodium and proton flux. Currently, halocin H6 is the only archaeocin that exerts a specific inhibitory effect on Na^+/H^+ exchanger (NHE) in both haloarchaean and mammalian cells (Lequerica et al. 2006). Therefore, it plays a major role in protecting the myocardium against ischemia and reperfusion injury (Besse et al. 2015). It further suggests the possibility that other archaeocins may have similar or different clinical applications. To realize their full clinical potential, it needs more research on their physical structures and their modes of action.

16.5 Conclusions and Future Perspectives

The metabolite diversity among haloarchaea has attracted immense interest during the last decade. Studies carried out in recent years have increased our knowledge of different aspects of halophilic Archaea, such as systemic and phylogenetic relationships, ecology and to a lesser extent physiology and genetics. Many of them have their origin in man-made hypersaline environment developed for various purposes, e.g., commercial solar salt, salted food, etc. Future research is necessary to address how the halophilic microorganisms originated during the early stage in the evolution of life and how they diversified and are distributed throughout the world. Their adaptiveness to both high salt concentration and temperature makes them sources of industrially valuable enzymes. Their biotechnological potential for producing compatible solutes, biopolymers, and other compounds is of industrial interest. Moreover, haloarchaea constitute an excellent model for study of adaptive mechanisms that permit them to tolerate multiple extreme conditions. Haloarchaea have developed strategies to survive under harsh conditions of pH, temperature, or

ionic strength. The knowledge on halocins and understanding how they act in such harsh environments represent an emerging domain of research. This field has been seldom explored until now. Halophilic bacteria and archaea generally use disulfide bond formation and ionic interactions as a main strategy to specifically balance and localize particular amino acids at the surface of the protein to stabilize their three-dimensional structures. Therefore, it would be interesting to investigate the specific properties of halocins which make them different from others that usually denatured under harsh conditions. Future research may clarify the different aspects of the production and roles of these halocins, biosynthesis, and maturation pathways, detailed structures and mechanisms of action, as well as their ecological roles. In order to realize their full clinical potential, further studies need to focus on their physical structure and modes of action so that clinicians will be able to predict which halocins have the desired pharmaceutical effect. It will also reveal its potential applications for mankind in the coming years.

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Microbial Diversity and Dynamics in Hydrocarbon Resource Environments

17

Ganshyam Prajapat, Shikha Jain, and Akhil Agrawal

Abstract

Hydrocarbon resource environments consist of oil reservoirs, oil sands, coal bed methane, and any other geological environment where fossil fuels are found. These reserves have diverse in situ physicochemical conditions where many indigenous microbial communities are present, if conditions are not too drastic for their growth and survival. The physical, chemical, and microbiological processes that govern the activity of these communities must be understood for optimal and economic exploitation. This chapter provides a comprehensive overview of the microbial ecology of oil reservoirs, oil sands, and coal bed methane. The diversity of several important groups such as sulfate-reducing and nitrate-reducing bacteria, methanogens, hyperthermophiles, and fermentative bacteria is discussed to illustrate the diverse and dynamic nature of the resident microbes.

Keywords

Sulfate-reducing bacteria · Nitrate-reducing bacteria · Methanogens · Fermentative bacteria · Oil reservoirs · Coal bed methane · Oil sands

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17.1 Introduction

Conventional hydrocarbon reserves consist of coal, oil, and natural gas that still provide the major portion of worldwide energy consumption. Unconventional hydrocarbon resource environments are those sources of oil and gas which require novel methods of extraction that are different from conventional methods. These consist of shale gas, oil sands, and coal bed methane reserves. Oil reservoirs are one of the major sources of hydrocarbons contributing more than 80% of the fossil fuel in the form of petroleum or crude oil. When the oil wells are drilled into these reservoirs, crude comes out due to in situ reservoir pressure; this is known as primary oil recovery. Oil produced by this recovery process is only 10% of the original oil in place (OOIP) (Planckaert 2005). For more oil production, reservoir pressure is rebuilt by the injection of water; this method is known as secondary oil recovery. This physical sweeping of oil by water can produce up to 40% of OOIP. The produced fluids from reservoirs are a mixture of water, oil, and gas, which has to be separated in three-phase separators (water, oil, and gas). The source of water depends on location of oil field. In the offshore oil field, water availability is not a problem because sea water can be used as the source water for secondary recovery. In the onshore (inland) oil field, water required for the flooding can be taken from surface water facilities. Due to strict governmental regulations and scarcity of surface water, the industries have to reuse the produced water for injection into the reservoirs. This is known as produced water reinjection (PWRI). Additional amount of water over PWRI is generally taken from municipal waste water, nearby lake, river, or underground water aquifer. When the oil to water ratio decreases, then oil industry either stops the production or adopts the tertiary recovery process. In this, chemical, thermal, miscible, and microbial mechanisms are used for enhanced oil recovery (EOR) (Mcinerney et al. 2005).

Crude oil is a complex mixture of hydrocarbons that are formed from fossilized buried organisms in underground sedimentary rocks and subjected to millions of years of geological transformations (Grigoryan et al. 2008; Speight 2014). The major classes of hydrocarbons are cycloalkanes, alkenes, and aromatics, whereas minor classes of hydrocarbons are non-hydrogenous compounds (N, S, and O organic compounds) and metals (nickel and vanadium) (Speight 1999; Wolicka and Borkowski 2012).

Oil is a complex mixture of organics, which has been biodegraded by indigenous microbial communities of the oil reservoirs anaerobically since geological times. Characterization of biodegradable oil is done by the Peters and Moldowan (PM) scale, which ranges from 0 to 10 based on the pattern of removal of specific compound classes. The higher PM value represents more degraded oil. API gravity is another scale used for the oil categorization where lower API gravity signifies heavy or highly degraded oil. Sequential degradation of oil begins with *n*-alkanes and alkylbenzenes in aliphatic and aromatic compounds, respectively. Most of the world oil reservoirs are rated as heavy or super heavy oil. Syntrophy between fermentative bacteria and methanogenic archaea aids in the degradation of oil in the absence of electron acceptors. Fermentative bacteria degrade oil into acetate and

CO_2 (unfavorable reaction), which are consumed by methanogenic bacteria to produce CO_2 and H_2. Mainly acetoclastic and hydrogenotrophic methanogenic processes lead to this bioconversion process (Head et al. 2010). This process is slow in reservoir conditions and can be enhanced by adding an electron acceptor during the secondary oil recovery process.

Oil sands or tar sands are loose or partially consolidated sandstone containing a mixture of clay sand, water, and extremely viscous form of crude or petroleum. These are unconventional type of petroleum deposits. During extraction of crude from oil sands, large volumes of waste mineral suspensions composed of wastewater, sands, silt, clay, and residual bitumen are left. This waste is stored in large ponds known as tailing ponds. These tailings are one of the most difficult environmental challenges facing the oil sands industry. The lack of knowledge and identification of individual compounds has become a major hindrance to the handling and monitoring of oil sands tailings. A better understanding of the chemical makeup and microbial communities can help in removing toxic components from these ponds. Oil sands tailing ponds have different microbial communities in various upper to lower layers. The detailed description of microbial communities found in these environments is given in later part of this chapter.

Coal bed methane (CBM) is a natural gas extracted from absorbed solid matrix of coal which is composed of methane and trace amount of ethane, nitrogen, carbon dioxide, and few other gases. The composition of CBM mainly depends on properties of coal. Deep CBM reservoirs are formed due to thermal coalification processes, whereas shallow CBM reservoirs are formed due to in situ microbial activity (Barnhart et al. 2016; Faiz and Hendry 2006; Ritter et al. 2015; Strąpoć et al. 2011).

The microbiology of CBM is related to fermentative, acetogenic, and methanogenic bacteria/archaea (Table 17.4). In CBM, initially, fermentative bacteria hydrolyze the fermentable complex to produce acetate, long-chain fatty acids, CO_2, H_2, NH_4^{+}, and HS^{-}. Produced H_2 and CO_2 are used by acetogenic bacteria to generate acetate; H_2, CO_2, and acetate are utilized by methanogens to produce methane. In CBM, methanogenic archaea present in the consortium and interspecies H_2 transfer are common (Zinder 1993).

A detailed account of the diversity and dynamics of microbial communities in these environments is given in following sections.

17.2 Microbiology of Oil Reservoirs and Oil Fields

Microbial activity is predominantly present everywhere, including oil reservoirs. In oil reservoirs, it is evidenced by biodegradation of oil. The indigenous anaerobic bacteria are mainly responsible for this process. The archaea and bacteria present in the oil reservoirs are physiologically very diverse. They belong to lithotrophic and heterotrophic groups like methanogens, fermentative, iron-reducing, nitrate-reducing, sulfate-reducing, and metal-reducing bacteria (Magot 2005; Magot et al. 2000; Voordouw et al. 1996).

In 1926, Edson S. Bastin and coworkers studied the microbial ecology of production waters for the first time (Bastin et al. 1926). In this study, they dealt with California and Illinois oil fields; the observations revealed that sulfate-reducing bacteria (SRB) are predominantly present and are indigenous to reservoirs. It was recorded for the first time that bacteria inhabit deep subsurface oil reservoirs. Since then many more investigations have been carried out which confirmed that subsurface environment is inhabited by various bacterial communities (Fredrickson et al. 1995; Parkes et al. 1994; Pedersen 2000). This is now widely accepted concept that indigenous bacterial communities are present in the subsurface environment. As deep surface environments are harsh and have drastic physical conditions, many of the microbial communities present there are not found elsewhere. For example, SRB species like *Desulfacinum infernum*, *Desulfacinum subterraneum*, *Desulfotomaculum halophilum*, *Desulfotomaculum thermocisternurn*, and *Desulfovibrio longus* are present in the oil field production water (Magot 2005). All these bacteria are native indigenous bacterial communities and have not been isolated from any other environment.

The culture-independent methods are now widely used for microbial community analysis. The use of these techniques confirmed that both archaea and bacteria are present in reservoirs. *Marinobacter*-, *Pseudomonas*-, and *Desulfovibrio*-related diverse groups of mesophilic bacteria and archaea were detected by rRNA sequencing. A mixture of thermophilic *Archaeoglobus fulgidus* and mesophilic *Aquabacterium* were detected by 16S rRNA gene sequence analysis of samples collected from North Sea oil reservoir (Brakstad and Lødeng 2005).

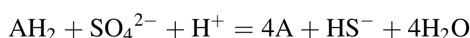
Many oil fields have similar species of bacteria that are not found anywhere; this is a strong evidence indicating that these bacteria are indigenous to oil reservoirs. The species of *Thermotoga* and *Thermoanaerobacte*r have been isolated from many oil fields worldwide, with the in situ temperatures between 60 and 80°C (Bonch-Osmolovskaya et al. 2003; Grassia et al. 1996; Magot 1996; Orphan et al. 2000; Slobodkin et al. 1999; Stetter et al. 1993b; Takahata et al. 2000). *Thermotoga elfii* and *Thermotoga subterranea* were isolated from Cameroon and the Paris Basin, France (Jeanthon et al. 1995; Ravot et al. 1995). Similarly, *Thermoanaerobacter* and *Thermoanaerobacterium* species have been isolated from various high-temperature and low-salinity reservoirs of Australia, Venezuela, Bahrain, and New Zealand (Grassia et al. 1996).

Temperature in deep oil reservoirs can exceed 130–150°C, which is beyond theoretical limit for life (Stetter et al. 1993b). For thermophilic microbes, maximum growth temperature is 121°C but can survive at 130°C (Kashefi and Lovley 2003). Microorganisms can inhabit reservoirs of depth up to 4000 m and in situ temperature of 120–130°C. The concentration of fatty acids increases in oil reservoir at 20–90°C and decreases above 90°C. These trends suggest that microbial activity is high at low temperatures and decreases beyond 80–90°C (Barth 1991; Carothers and Kharaka 1978; Fisher 1987). *Archaeoglobus* and *Thermococcus* species were isolated at 85 and 102°C, respectively, from the Thistle oil fields (Stetter et al. 1993a).

The details of different microbial communities present in oil reservoirs are provided in the following sections.

17.2.1 Sulfate-Reducing Bacteria (SRB) and Archaea

Sulfate-reducing bacteria and archaea together are known as sulfate-reducing microorganisms (SRMs), which are obligate anaerobic prokaryotes that reduce sulfate into sulfide (Birkeland 2005). These bacteria use various organic compounds as an electron acceptor. The reaction of the process known as dissimilatory sulfate reduction (DSR) is as follows:



$$AH_2 + SO_4{}^{2-} + H^+ = 4A + HS^- + 4H_2O$$

SRM plays a role in global sulfur cycle that occurs in almost all anaerobic environments. These microorganisms either completely oxidize the organic acids into CO_2 or incompletely to acetate. They commonly use lactate, ethanol, acetate, propionate, and various other fatty acids. Some autotrophic strains use CO_2 as a C source and H_2 as electron source. Other than sulfate, SRM uses sulfite, thiosulfate, and sulfur as electron acceptors.

The classification of SRMs is traditionally based on the morphology, physiology, and biochemical characteristics. They have diverse morphology like vibrio, oval, rod, sphere, and curved rods. SRMs that oxidize the organic material completely into CO_2 are known as complete oxidizers, while incomplete oxidizers can oxidize the organic compounds to acetate. Complete oxidizers are able to use a variety of substrates including fatty acids and aromatic compounds, while incomplete oxidizers do this rarely.

Sulfate reduction is a three-step process. In the last step, sulfite is converted to sulfide with transfer of six electrons to sulfite. This reaction is carried out by the dissimilatory sulfite reductase (Dsr). According to optical properties, Dsr enzyme is of several types like desulfoviridin, desulforubidin, desulfofuscidin, and P582. Specific absorption spectrum is the key characteristic for the taxonomical classification of SRM. Siroheme and iron sulfur center (Fe-S) is responsible for optical properties of Dsr. The structural variation and its composition in these groups lead to spectral differences.

Majorly 40 genera of SRB belonging to four lineages, (i) δ-proteobacteria, (ii) Gram-positive bacteria of the genera *Desulfotomaculum* and *Desulfosporosinus*, (iii) thermophilic Gram-negative bacteria of the genus *Thermodesulfobacterium*, and (iv) thermophilic Gram-negative bacteria of the genera *Thermodesulfovibrio* and *Thermodesulfobium*, are involved in SRM. Archaea that belong to SRM are hyperthermophilic in nature. They are *Archaeoglobus* spp. (*A. fulgidus*, *A. profundus*, and *A. veneficus*) from Euryarchaeota phylum and *Caldivirga maquilingensis* from Crenarchaeota.

Hyperthermophilic SRMs were isolated from North Sea and Alaskan oil reservoirs (Stetter et al. 1993b). *Archaeoglobus* (enriched at 85°C) isolated from both North Sea and Alaskan oil fields were cocci. *Thermotoga* (rod shaped, enriched at 85°C) isolated from Alaskan oil field was with coccoid archaea; *A. lithotrophicus* cells showed wing-like ultra-flat protuberances and high surface area to volume ratio that helps in gas exchange (H_2 utilization).

SRB have been widely studied for anaerobic metabolism, corrosion of metals, oil field souring, etc. In nature, SRB thrive where sulfate, O_2-limiting condition, and settled organic particles are present and decomposition of organic matter takes place (Cord-Ruwisch et al. 1987). SRB were isolated from brackish water, freshwater sediments, anaerobic muds, marine sludge, and rumen content (Widdel et al. 1983; Widdel and Pfennig 1977). Widdel et al. (1983) isolated three SRB strains (2 ac9, 3 ac10, and 4 ac11) requiring salt and acetate for their growth (Widdel et al. 1983). Generally SRB are indigenous community of microorganisms in anaerobic zones like oil field production facilities (Cord-Ruwisch et al. 1987). The injection well plugging by FeS flocs in northern Germany oil fields was related to SRB, and it was concluded that crude oil processing unit was the main source of sulfide in eastern Germany oil field (Cord-Ruwisch et al. 1987). In oil industries, SRB were isolated from oil reservoirs, injection water, production water, aboveground facilities, transport pipelines, and oil storage tanks. Strain Hxd3 (tiny cells 0.4–0.5 × 0.8–2 μm) was isolated from precipitates of oil-water separators of an oil field near Hamburg (Aeckersberg et al. 1991). This strain was able to grow anaerobically on hexadecane. Thermophilic spore-forming SRB related to *Desulfotomaculum* sp. (43–78°C) were isolated from oil fields placed in Norwegian sector of the North Sea (Rosnes et al. 1991). These SRB grow on a wide variety of fatty acids and alcohols. Spores were extremely resistant to high temperature (131°C for 20 min). The δ-proteobacteria SRB lineages are in two families: Desulfovibrionaceae and Desulfobacteraceae (Devereux et al. 1990; Widdel and Bak 1992). Both families are in one division from the same common ancestral phototrophic proteobacterium (Woese 1987). The genera *Thermodesulforhabdus*, *Desulfacinum*, *Desulfobulbus*, *Desulfocapsa*, *Desulfofustis* and *Desulforhopalus* belong to Desulfobacteraceae. The genus *Desulfovibrio* has been used as model organism for understanding the mechanism of dissimilatory sulfate reduction.

Pyrococcus sp. that grows at 60–103°C was also isolated from well head samples of North Sea oil field (Stetter et al. 1993b). *Desulfomicrobium* and *Desulfovibrio* species were isolated from oil production facilities of western Canadian oil fields (Armstrong et al. 1995). Thermophilic SRB *Desulfotomaculum thermoacetoxidan*, *Desulfacinum infernurn*, and *Thermodesulforhabdus norvegicus* were isolated from North Sea oil. *Desulfovibrio*-type strains (strain G3 100, G4 100, G3 BE, and G4 BE) were isolated from crude oil storage tanks and production water of southern Vietnam oil field (Nga et al. 1996). SRB isolated from France, North Sea, and Gulf of Guinea belong to *Desulfovibrio* and *Desulfomaculum* (Tardy-Jacquenod et al. 1996). Another strain *Desulfotomaculum halophilum* SEBR 3139 was isolated from oil field brine of France (Tardy-Jacquenod et al. 1996). *Desulfovibrio capillatus* (Topt 40°C) was isolated from a Mexican oil field separator (Miranda-Tello et al. 2003). The strain C4, isolated from Coleville oil field water sample enrichments, is the closest relative of *Desulfomicrobium norvegicum* (sulfate reducer, acetate oxidizer; δ-proteobacteria) or *Desulfovibrio desulfuricans* (uses both sulfate and nitrate) (Hubert and Voordouw 2007).

Kaster et al. (2007) studied the effect of nitrate and nitrite on sulfide production by using two thermophilic sulfate-reducing enrichments from oil fields of North Sea

(Kaster et al. 2007). Sulfate enrichments, NS-tSRB1 and NS-tSRB2, were grown with acetate-propionate-butyrate and lactate as electron donors, respectively. In these enrichments, *Thermodesulforhabdus norvegicus* (in NS-tSRB1) and *Archaeoglobus fulgidus* (in NS-tSRB2) were present. In both enrichments, nitrate (10 mM) has no effect on sulfide production, while nitrite (0.25 mM or higher) inhibited sulfate reduction (Kaster et al. 2007). Gorigoryan et al. (2008) studied the microbial community of upflow and packed-bed bioreactors inoculated with the consortium of SRB from Neuque'n Basin oil field in the western Argentina (Grigoryan et al. 2008). The genera *Desulfobotulus*, *Desulfotignum* and *Desulfobacter* (SRB genera), and *Desulfuromonas* (sulfur reducing) were detected in VFA and sulfate enrichments.

Agrawal and Lal (2009) used DGGE-PCR-based approach of *dsr*B gene for enumeration of SRB. Based on different DGGE band patterns, they concluded that *Desulfotomaculum aeronauticum*, *Desulfotomaculum hafniense*, *D. thermoacetoxidans*, *Desulfovibrio vulgaris* like sequences are present in Indian oil fields (Agrawal and Lal 2009). Kaur et al. (2009) studied Kathloni oil field (Assam, India) and found the culturable sulfide-producing bacterial species were *Anaerobaculum mobile*, *Garciella nitratireducens*, *Clostridium sporogenes*, *Thermosediminibacter oceani*, *Coprothermobacte*r sp., *Thermodesulfovibrio* sp., *Thermodesulfobacterium* sp., *Thermodesulfotobacterium* sp., and *Caldanaerobacter* sp. (Kaur et al. 2009). These species use VFAs such as acetic, propionic, isobutyric, butyric, and isovaleric acids. The mesophilic culturable sulfidogenic communities of production water of onshore oil fields (North-Eastern India) were also studied (Agrawal et al. 2010). Isolated SRB were related to phylum Proteobacteria (*Desulfovibrio*, *Desulfomicrobium*, and *Desulfobulbus*) and *Desulfotomaculum* (Gram-positive SRB). Thiosulfate-reducing bacteria belong to the phylum Firmicutes (*Clostridium* and *Fusibacter*) and Proteobacteria (*Enterobacter* and *Citrobacter*). *Clostridium* (69%) and *Desulfovibrio* (53%) were dominant communities in the production fluids (Agrawal et al. 2010). Callback et al. (2011) simulated low-temperature oil field of Canada in the laboratory (Callbeck et al. 2011). 16S rRNA gene sequence analysis showed the presence of sulfate reducer *Desulfobulbus* spp. dominant in deep side of the column.

Zapata-Peñasco et al. (2013) studied Mexican oil field for the SRB and corrosive bacteria by analyzing functional *dsr*AB gene and *nif*D gene (nitrogenase gene) sequences, respectively (Zapata-Peñasco et al. 2013). They found the presence of the orders Desulfovibrionales, Desulfobacterales, and Desulfuromonadales. The *dsr*AB gene phylogenetic analysis showed the presence of *Desulfomicrobium*, *Desulfovibrio*, *Desulfohalobium*, *Desulfococcus*, *Desulfosarcina*, *Desulfobacter*, *Desulfobacterium*, and *Desulfobulbus*, while *nif* D gene phylogenetic analysis showed the presence of Fe (III)-reducing bacteria (Desulfuromonadales) such as *Desulfuromusa*, *Pelobacter*, *Malonomonas*, and *Desulfuromonas*. The Barrancas field (80°C) and the Chihuido de la Salina (CHLS) field of Argentina (65–70°C) were flooded with high sulfate-containing injection water (Agrawal et al. 2014). Sulfate was not reduced to sulfide by SRB at downhole, while sulfate reduction occurred in the aboveground facilities (low temperature). In a water plant of

Barrancas field, the presence of *Thiomicrospira* (85%, so-NRB, Gammaproteobacteria) led to reoxidation of sulfide into sulfate. Tian et al. (2017) showed that *Desulfotignum*, *Desulfotomaculum*, *Desulfovibrio*, *Desulfobulbus*, and *Desulfomicrobium* were abundant in Xinjiang reservoir blocks (LZ, QZ, and DQ block) (Tian et al. 2017). In low-temperature reservoir, *Desulfovibrio*, *Desulfomicrobium*, *Thioclava*, and *Sulfurimonas* were abundant, while in high-temperature reservoir, *Desulfotomaculum*, *Desulfotignum*, *Thiobacillus*, and *Dechloromonas* were dominant (Tian et al. 2017).

Biochemistry of Sulfate Reduction in SRB During anaerobic respiration by SRM, sulfate is reduced into sulfide with eight electron transfer. This reaction requires several enzymes and various intermediates. Sulfate reduction is the three-step process. In the first step, sulfate is converted to adenosine-5-phosphosulfate (APS) with use of one ATP by ATP sulfurylase; the produced pyrophosphate is further converted to phosphate by pyrophosphatase, and the formation of phosphate favors the overall APS formation. In the second step, the conversion of APS into sulfite and AMP takes place by the action of APS reductase, APS is an electron acceptor, and NADH is an electron donor (Chen et al. 1994). In the third step, sulfite is converted to hydrogen sulfide by the action of dissimilatory sulfite reductase (Dsr). This reduction process requires six electrons, which come from H_2 oxidation. By oxidation of H_2 by periplasmic hydrogenase, electrons are released into periplasmic cytochrome c3. Cytochrome c3 transfers these electrons to high molecular mass cytochrome c (Hmc). The Hmc is the part of membrane integral protein component. Electrons are transferred from integral subunits to Fe-S cluster containing subunits of Hmc and further transferred directly to APS or Dsr or may involve other electron carriers. Electrons are transferred from transmembrane protein to the electron acceptor and chemiosmatically synthesize ATP by creating membrane potential and proton gradient.

SRM uses various types of organic acids, but lactate is the most important substrate that is utilized by majority of the SRM. Lactate is converted to pyruvate by membrane-bound lactate dehydrogenase and further converted into acetyl-coenzyme-A. During incomplete oxidation of lactate, it is converted to acetate and performs substrate-level phosphorylation of ADP to ATP. In hydrogen cycling model, H_2 is produced from lactate oxidation in the periplasmic space by periplasmic hydrogenase. If complete oxidation takes place, further acetate oxidation takes place by two mechanisms: modified citric acid cycle and carbon monoxide dehydrogenase (CODH) pathway.

Cultivation-dependent techniques are used to enumerate SRM in water samples collected from oil reservoir. Low to high temperature decreases SRM population (Nazina et al. 1995), while salinity of water creates differentiation in SRM community (Voordouw et al. 1992). Culture-independent techniques are used for the detection and enumeration of SRM in produced water samples by genus-specific antibodies, oligonucleotide microchip method, cloning and sequencing of 16S rRNA gene, and reverse sample genome probing.

17.2.2 Methanogens

Methanogens are described in the Archaea domain. Methanogenesis in oil fields has been reported in the early 1950s. Different types of methanogens were isolated from oil reservoirs having mesophilic temperature and low to high salinity. Most of the methanogens isolated from oil fields belong to Euryarchaeota phylum (Jeanthon et al. 2005). *Methanobacterium bryantii* is a hydrogenotrophic rod-shaped archaeon isolated from Romashkinskoe oil field (Davydovacharakhchyan et al. 1992). Another one *Methanobacterium ivanovii* has been isolated from Bondyuzskoe oil fields, Tatarstan (Belyaev et al. 1986).

Generally the abundance of methanogens decreases with increasing salinity. *Methanocalculus halotolerans* has the ability to grow up to 12.5% NaCl (Ollivier et al. 1997). Methylotrophic methanogen such as *Methanococcoides euhalobius*, isolated from Tatarstan (Davidova et al. 1997), is a halophilic methanogen (grows maximally at 140 g/l salt) that uses methylamines and methanol and grows when calcium and magnesium ions are present. Still another archaeon *Methanosarcina siciliae*, isolated from the Gulf of Mexico, also has the ability to use methanol and methylamine (Ni and Boone 1991). Another acetoclastic methanogen *Methanosarcina mazei* strain 47 also produces methane from methanol and methylamine (Obraztsova et al. 1987).

Methanogens isolated from high-temperature oil reservoirs are mostly hydrogenotrophs. *Methanobacterium thermoautotrophium* is commonly present in moderate saline stratal water. This strain is thermophilic and halophilic in nature, isolated from a virgin oil reservoir of Southern California (Ng et al. 1989). *Methanothermococcus thermolithotrophicus* was isolated from the oil platform of Statfjord field of North Sea (Nilsen and Torsvik 1996).

Acetogenic methanogens of the genus *Methanosaeta* was present in deep side of the oil field simulating sand-packed column after nitrate treatment (Callbeck et al. 2011). The microbial community of oil enrichment culture and sand-packed column was studied for low-temperature oil reservoirs (Berdugo-Clavijo and Gieg 2014). Results of pyrosequencing showed that archaeal numbers increased in column (63%) by enrichment. In the original enrichment, Euryarchaeota, Spirochaetae, Firmicutes, and Proteobacteria dominated, while in column communities, Euryarchaeota, Proteobacteria, and Actinobacteria were dominant; Firmicutes and Spirochaetae were less. At genus level, *Smithella* (27%), *Methanosaeta* (25%), and *Methanoculleus* (23% total reads) were present in enrichment, and *Methanobacterium* (56%) and *Pseudomonas* (16%) were dominant in the column (Berdugo-Clavijo and Gieg 2014).

17.2.3 Fermentative Microorganisms

In oil reservoirs, three types of fermentative bacteria are present: mesophilic, thermophilic, and hyperthermophilic. Fermentative bacteria gain energy by substrate-level phosphorylation of available organic compounds such as sugars, peptides,

amino acids, and other organic acids (Ollivier and Cayol 2005). They can also use inorganic compounds like thiosulfate and nitrate as an electron acceptor for oxidization of substrate when these are present. In the absence of any electron acceptor, these bacteria purely work as fermentative bacteria and can also survive in different salinity ranges.

17.2.3.1 Mesophilic Fermentative Bacteria

Mesophilic fermentative anaerobic bacteria isolated from oil reservoir can survive in low to moderate saline conditions. The genus, which is moderately halophilic, isolated from petroleum reservoirs is *Haloanaerobium*. They grow optimally at 2.5% (maximum 10%) NaCl (Gevertz et al. 1991). These are saccharolytic organisms (can use mono and disaccharides) and produce H_2, CO_2 and C_2 compounds like acetate and ethanol. *H. ethylicum* (Rengpipat et al. 1988), *H. salsuginis* (Bhupathiraju et al. 1994), *H. congolense* (African oil fields in Congo), and *H. kushneri* (Bhupathiraju et al. 1999) were isolated from Southeast Vassar Vertz Sand Unit (Bhupathiraju et al. 1991; Bhupathiraju and Knapp 1993). These strains produce acids, solvents, and gases from carbohydrate degradation and can be utilized as candidate for microbially enhanced oil recovery (MEOR). Other mesophilic fermentative bacterium, *Spirochaeta smaragdinae*, was isolated from African offshore oil fields in Congo (Magot et al. 1997a), which uses thiosulfate as a terminal electron acceptor and capable of fermenting sugars into lactate, ethanol, H_2, and CO_2 (Miranda-Tello et al. 2003). *Dethiosulfovibrio peptidovorans* has been isolated from corroded offshore oil fields of Congo (Magot et al. 1997b) that ferments peptone and amino acids but not sugars. It causes corrosion when reduces thiosulfate into sulfide. Another genus *Fusiobacter paucivorans* was isolated from the oil reservoir of Africa, which is able to reduce thiosulfate and elemental sulfur into sulfide (Ravot et al. 1999). It uses cellobiose, glucose, mannitol, and ribose as energy sources, with butyrate, acetate, H_2, and CO_2 as the metabolic end products. From formation waters of Tartaria and Siberia oil fields, anacetogenic anaerobe *Acetoanaerobacterium romashkovii* was isolated (Davydovacharakhchyan et al. 1992). This strain uses H_2 and CO_2 as sole carbon source.

17.2.3.2 Thermophilic Fermentative Bacteria

Most of the thermophiles isolated from high-temperature oil reservoirs belong to the order Thermotogales. This order includes genera *Thermotoga*, *Thermosipho*, *Fervidobacterium*, *Petrotoga*, *Geotoga*, and *Marinitoga*. The four genera *Thermotoga*, *Thermosipho*, *Petrotoga*, and *Geotoga* were detected by both molecular and culture-based techniques (Bonch-Osmolovskaya et al. 2003; Magot et al. 2000; Orphan et al. 2000; Takahata et al. 2000). *Thermotoga* and *Thermosipho* species were isolated from various oil fields as well as environments like thermal springs and hydrothermal vents. However, *Petrotoga* and *Geotoga* spp. have been isolated only from oil reservoirs (Davey et al. 1993; Magot et al. 2000) and are considered indigenous to oil reservoirs. Strains of *Thermotoga* that are identified from various oil reservoirs are *T. elfii* (Ravot et al. 1995), *T. subterranean* (Jeanthon et al. 1995), and *T. hypogea* (Fardeau et al. 1997), *T. petrophila*, and *T. naphtophila*

(Takahata et al. 2001). *Geotoga petraea*, *Geotoga subterranea*, and *Petrotoga miotherma* were isolated from Oklahoma and Texas oil reservoirs (Davey et al. 1993). Another species of *Petrotoga*, *P. mobilis*, was isolated from North Sea oil fields (Lien et al. 1998); *P. olearia* and *P. sibirica* were isolated from Siberian continental oil reservoirs (L'Haridon et al. 2002), and *P. mexicana* was isolated from Gulf of Mexico (Miranda-Tello et al. 2004). *Thermosipho* species, *T. geolei*, was isolated from continental reservoir in western Siberia, Russia. Genera *Thermoanaerobacter* and *Thermoanaerobacterium* were isolated from hot and slightly saline reservoirs (Grassia et al. 1996). *Thermoanaerobacter* sp. (L'haridon et al. 1995), *T. brockii* (Cayol et al. 1995) and *T. subterraneus* (Fardeau et al. 2000), were isolated from French continental oil reservoir. *T. subterraneus* reduces sugars into acetate and L-alanine, but in the presence of thiosulfate, L-alanine production is decreased and acetate production increased. *T. yonseiensis*, *T. tengcongensis*, and *Carboxydibrachium pacificum* metabolize glucose to produce acetate and L-alanine with reduction of thiosulfate only (not the sulfate). A Gram-positive, anaerobic, and moderate thermophilic strain *Mahella australiensis* was isolated from Australian oil reservoir. It ferments different types of sugars (Salinas et al. 2004b).

Besides *Thermotogales* and *Thermoanaerobiaceae*, other fermentative bacteria have also been isolated from oil field environments. *Anaerobaculum thermoterrenum* was isolated from Redwash oil fields in Utah (Rees et al. 1997). This strain grows not only in sugars but also in organic acids (citric, fumaric, and tartaric acids). This strain uses both sulfur and thiosulfate as an electron acceptor.

By dot blot hybridization studies, *Thermococcus littoralis*, *Thermococcus celer* (grow up to 80°C), and *Pyrococcus* sp. (grows up to 102°C) were identified (Stetter et al. 1993b). These archaea are pleomorphic coccoid cells and use a variety of energy sources like peptides or carbohydrates during fermentation. *Pyrococcus furiosus* ferments maltose and produces L-alanine (Kengen and Stams 1994; Schönheit and Schäfer 1995). Another species, *Thermococcus sibiricus*, isolated from oil reservoir of western Siberia is also able to utilize peptides (Miroshnichenko et al. 2001). There are some beneficial effects of the presence of fermentative microorganisms in the oil reservoir, like production of gases and exopolysaccharides. For example, *T. maritima* and *T. litoralis* are able to produce exopolysaccharides (Rinker and Kelly 2000). But when they metabolize thiosulfate, sulfate or elemental sulfur, it leads to souring and corrosion problems.

17.2.4 Nitrate-Reducing Microorganisms

Nitrate-reducing bacteria (NRB) use nitrate as a terminal electron acceptor and play a major role in nitrogen cycle. They convert nitrates and nitrites into nitrogen-containing gases. Generally, in oil reservoirs, NRB are present in low abundance. This low abundance of NRB in reservoirs is due to the absence or low concentration of nitrate in reservoirs. NRB are efficient to combat the souring problem. To stimulate NRB activity, nitrate is injected artificially into the reservoirs. NRB ceases the sulfide production by following mechanisms: (1) bio-competitive exclusion for

Fig. 17.1 Different inhibition mechanisms of sulfide production in oil fields during nitrate injection. (1) Bio-competitive exclusion for same oil organics between sulfate-reducing bacteria (SRB) and heterotrophic nitrate-reducing bacteria (hNRB), (2) sulfide oxidation by sulfide-oxidizing nitrate-reducing bacteria (so-NRB), (3) dissimilatory bisulfite reductase (Dsr) enzyme inhibition by produced nitrite by so-NRB and hNRB, and (4) increased redox potential by nitrate that inhibits SRB growth

the same organics, (2) by the activity of sulfide-oxidizing and nitrate-reducing bacteria (so-NRB) that oxidize sulfide into sulfate, (3) modification of redox potential or nitrite production by NRB, and (4) inhibition of Dsr enzyme by nitrite (Davidova et al. 2001; Gieg et al. 2011; Hubert et al. 2003). These mechanisms are presented in Fig. 17.1.

In oil reservoirs, NRB can be microaerobic, aerobic, facultative anaerobic or aerobic (Myhr and Torsvik 2000; Telang et al. 1997; Voordouw et al. 1996). NRB follows metabolism either hydrogen oxidization (hydrogenotrophic) (Gevertz et al. 2000; Kodama and Watanabe 2003) or heterotrophic (uses organic acids). The so-NRB oxidize sulfide into sulfate or elemental sulfur and reduce nitrate to nitrite or N_2. Two so-NRB species CVO and FWKO B were isolated from Coleville oil field in Saskatchewan, Canada (Gevertz et al. 2000). These strains are microaerophilic and chemolithotrophic when H_2, formate, and sulfide are present. Strain CVO completely reduces the nitrate into nitrous oxide and then into N_2, while strain FWKO B partially reduces nitrate into nitrite. Other strain YK-1, isolated from oil storage cavity in Kuji, Japan, had similar metabolic features to CVO and FWKO B (Kodama and Watanabe 2003). Different heterotrophic NRB were also isolated from oil reservoirs. Mesophilic *Denitrivibrio acetiphilus* was isolated from oil field simulating columns (Myhr and Torsvik 2000). This NRB is anaerobic, uses nitrate as a terminal electron acceptor and also ferments fumarate. Thermophilic heterotrophic NRB *Graciella nitratireducens* was isolated from Gulf of Mexico (Miranda-Tello et al. 2003). Both *Denitrivibrio acetiphilus* and *Graciella nitratireducens* have the ability to reduce the nitrate into ammonium. Another strain isolated from an Australian oil well was *Petrobacter succinatimandens* (Salinas et al. 2004a). This strain may use oxygen or nitrate as a terminal electron acceptor and oxidizes organic acids. *Marinobacter aquaeolei* was isolated from an offshore platform in southern Vietnam (Huu et al. 1999). This strain has the ability to grow on hydrocarbons aerobically and also uses acetate and citrate in the presence of nitrate. Two other hydrocarbon oxidizers of *Geobacillus* species (aerobic thermophiles) were isolated from the oil reservoir in Russia (Nazina et al. 2001). One is *Geobacillus*

subterraneus that reduces nitrate to N_2 and the other is *Geobacillus uzenensis* that reduces nitrate to nitrite only.

Hubert et al. (2009) isolated SRB, NRB, and so-NRB from the produced water collected from the Coleville oil field of western Canada. Six NRB strains, NO3B, N2, C4, C6, KW, and NO2B and two so-NRB strains were isolated (Hubert and Voordouw 2007). So-NRB strains had similarity with *Thiomicrospira* sp. strain CVO and *Arcobacter* sp. strain FWKO B sequences. Authors suggest that these bacteria are indigenous to reservoir because similar organisms were also isolated from other nearby oil fields and similar environments. Strains C6, NO2B, and KW belong to subdivision Epsilonproteobacteria. These strains were found closest to cultivated organisms *Sulfurospirillum deleyianum* (perform dissimilatory nitrate reduction to nitrate and ammonia). Strain KW performs nitrate reduction and also couples the reduction of nitrate with oxidation of sulfide into sulfur or polysulfide. Metabolic properties of other strains, C6 and NO2B, were very similar to strain KW. The *Thauera* sp. strain N2 (betaproteobacteria) performed complete reduction of nitrate into N_2 (Hubert and Voordouw 2007). *Paracoccus* sp. strain NO3B had similarity with cultivated *Paracoccus denitrificans* (alpha-proteobacteria). Bødtker et al. (2008) studied the long-term effect of nitrate treatment on oil fields (Bødtker et al. 2008). Veslefrikk and Gullfaks oil fields (North Sea) were treated with a low concentration of nitrate (0.23–0.33 mM). They analyzed the microbial community of these treated oil fields and found that so-NRB *Sulfurimonas* was abundant throughout the treatment period (Bødtker et al. 2008).

Canadian oil fields are inhabited by heterotrophic NRB and so-NRB (Cornish Shartau et al. 2010). *Sulfurospirillum* and *Denitrovibrio* perform nitrate reduction to ammonium. *Sulfurimonas*, *Arcobacter*, and *Thauera* perform denitrification. Besides these, *Candidatus Brocadia* and *Candidatus Kuenenia* were present which perform anammox reaction (decrease the ammonium concentration in a reservoir) (Cornish Shartau et al. 2010). In oil field simulating column, *Arcobacter* and *Pseudomonas* were found at the bottom after nitrate treatment (Callbeck et al. 2011). Feng et al. (2011) studied Shengli and Huabei oil fields of China (60°C) for nitrate-reducing microbial community analysis by using amplified *nap*A gene fragments as a biomarker (Feng et al. 2011). They identified bacteria belonging to orders Rhodocyclales and Burkholderiales from production fluid. In this study they combine both molecular and culture-dependent approaches for the evaluation of nitrate-reducing community in oil reservoir by using *nap*A gene (Feng et al. 2011).

The non-hydrogenotrophic NRB *Prolixibacter* sp. strain MIC1-1 (order *Bacteroidales*) was isolated from a crude oil sample of oil well in Akita, Japan (Iino et al. 2015). This strain grows on pyruvate as an organic source, nitrate as an electron acceptor and Fe^0 as an electron donor. This is the first identified strain belonging to *Bacteroidetes* that has role in corrosion of Fe^0. The *Thauera* sp. strain TK001 and *Pseudomonas* were dominating mesophilic NRB. *Petrobacter* sp. strain TK002 and *Geobacillus* sp. TK003 were present in the consortia of Canadian oil field samples (Fida et al. 2016).

17.2.5 Iron-Reducing Microorganisms

In the oil reservoir rocks, iron is present in the form of a ferric oxide (Slobodkin et al. 1999). Iron-reducing bacteria *Alteromonas putrefaciens* (Nazina et al. 1995; Semple and Westlake 1987) and *Deferribacter thermophiles* (isolated from Beatrice oil fields) (Greene et al. 1997) are the mesophilic iron-reducing bacteria isolated from oil reservoirs. *Deferribacter thermophiles* is a Gram-negative, halophilic anaerobe with the ability to reduce various inorganic compounds such as manganese (IV), iron (III), and nitrate with the presence of yeast extract, peptone, casamino acids, tryptone, hydrogen, malate, acetate, citric acid, pyruvate, lactate, succinate, and valerate. *Alteromonas putrefaciens is* also capable of reducing elemental sulfur, sulfite, and thiosulfate into sulfide (Moser and Nealson 1996). It uses H_2 or formate as an electron donor and iron oxyhydroxide as an electron acceptor (Nealson 1994). Bacteria *Thermotoga subterranean* SLIT, *Thermotoga maritima* M12597, *Thermoanaerobacter acetoethylicus* SL26 and SL28, and *Thermoanaerobacter brockii* M739 and archaea *Thermococcus* T642, T739, and T13044 are able to reduce ferric iron as terminal electron acceptor with oxidization of hydrogen and peptone (Slobodkin et al. 1999).

17.2.6 Low-Temperature Reservoir Microbial Community

Very few investigations have been carried out on analyzing microbial communities of low-temperature reservoirs (Table 17.1). Voordouw et al. (1996) used 16S rRNA gene sequencing for understanding the microbial community composition of low-temperature oil reservoir. They found the presence of aerobic mesophilic bacteria *Arcobacter*, *Thiomicrospira*, and *Oceanospirillum* in oil field samples (Voordouw et al. 1996). Later on, Grabowski et al. (2005) used both culture-dependent and molecular approaches for understanding the microbial diversity of low-salinity, low-temperature, and non-water-flooded oil reservoirs of Pelican Lake, western Canada (Grabowski et al. 2005). They successfully identified sulfate reducers, acetoclastic methanogens, and denitrifying bacteria by the culture-dependent technique. The class Firmicutes, Deltaproteobacteria, Epsilonproteobacteria, and Spirochaetia and Euryarchaeota were dominantly present. The 16S rRNA gene clone libraries and sequencing results showed the presence of archaeal orders Methanosarcinales and Methanomicrobiales. They concluded that methanogenesis was a terminal process in the investigated oil reservoir, and both acetoclastic and lithotrophic methanogens were present. By the culturable approach, *Acetobacterium carbinolicum*-related organism (homoacetogenic bacterium) and lactate-utilizing SRB belonging to the genera *Desulfomicrobium* and *Desulfovibrio* were isolated. Aerobic bacteria related to *Thauera phenylacetica* (98% identity) and *Tannerella forsythensis* (88% identity) and fermentative bacterium *Propionicimonas paludicola* were also isolated (Grabowski et al. 2005). Agrawal et al. (2012) studied low-temperature Medicine Hat Glauconitic C (MHGC) field, Alberta, Canada, treated with nitrate for souring treatment (Agrawal et al. 2012). Microbial

Table 17.1 Microbial communities present in low-temperature oil fields

S. no.	Oil field name	Bacterial communities	References
1.	Western Canada oil field	*Arcobacter*, *Thiomicrospira*, and *Oceanospirillum*	Voordouw et al. (1996)
2.	Pelican Lake oil field, western Canada	*Acetobacterium carbinolicum*, *Desulfomicrobium*, *Desulfovibrio*, *Thauera* sp., *Tannerella* sp., *Propionicimonas* sp., Methanosarcinales, and Methanomicrobiales	Grabowski et al. (2005)
3.	Medicine Hat Glauconitic C (MHGC) field, Alberta, Canada	*Methanoculleus*, *Methanosaeta*, *Methanolinea*, *Methanocalculus*, NRB: *Pseudomonas*, *Thauera*, *Azarcus*, *Arenimonas*, *Comamonas*, *Rhodocyclaceae*, *Desulfobacteria*, *Desulfovibrio*, *Smithella*, *Kosmotoga*. *Clostridium*, Comamonadaceae, *Flavobacterium*, *Methanoculleus*, *Methanolinea*, and Methanosarcinales	Agrawal et al. (2012) and Folarin et al. (2013)
4.	Enermark oil field, Alberta, Canada	*Acetobacterium*, *Thalassolituus*, *Rhodococcus*, *Sphingomonas*, *Brevundimonas*, *Stappia* *Methanolobus*, and *Methanobacterium*	Kryachko et al. (2012)
5.	Xinjiang oil field, China	*Sphingomonas*, *Azospirillum*, *Paracoccus*, *Ochrobactrum*, *Alcanivorax*, *Hydrogenophilaceae*, *Rhizobium*, *Arcobacter*, *Halomonas*, *Spirochaeta*, *Bacteroides*, *Pseudomonas*, *Ochrobactrum*, *Acinetobacter*, *Marinobacter*, *Methanoculleus*, *Methanosaeta*, *Methanocorpusculum*, and *Methanocalculus*	Gao et al. (2014 2015)
6.	Nigerian oil fields	Porphyromonadaceae, Deferribacteraceae, *Parvibaculum*, *Thalassobaculum*, *Rhodobacteraceae*, Rhodospirillaceae, *Marinobacter*, *Desulfovibrio*, *Desulfuromonas*, *Geoalkalibacter*, *Methanolobus*, and *Methanosaeta*	Okoro et al. (2014)
7.	Canadian oil field	*Ralstonia*, *Arcobacter*, *Sulfurospirillum*, *Methanohalophilus*, *Methanocalculus*, *Methanolobus* sp., *Sulfurospirillum*, *Methanolobus*, *Pelobacter*, and *Bacillus* sp.	Kryachko et al. (2017)

community analysis of oil field samples of 16S rRNA gene showed the presence of methanogens *Methanoculleus*, *Methanosaeta*, *Methanolinea*, and *Methanocalculus*; NRB, *Pseudomonas*, *Thauera*, *Azarcus*, *Arenimonas*, *Comamonas*, and

Rhodocyclaceae; SRB, *Desulfobacteria* and *Desulfovibrio*; and others like syntroph *Smithella* and *Kosmotoga* (Agrawal et al. 2012).

Kryachko et al. (2012) studied the microbial composition associated with oil and water of same MHGC oil field, Canada (Kryachko et al. 2012). Biomass was separated from water and oil individually. Separated biomass was subjected to DNA isolation and further sequencing. The observations suggested that water-associated microbial community was more diverse than oil-associated community. Oil-associated community was represented by hydrogenotrophic methanogens (*Methanolobus* and *Methanobacterium*) and acetogen (*Acetobacterium*). The organisms present in oil phase showed that oil is the major source of hydrogen. Other than methanogens, Gammaproteobacteria (genus *Thalassolituus*), Actinobacteria (genus *Rhodococcus*), and Alphaproteobacteria (genera *Sphingomonas*, *Brevundimonas*, and *Stappia*) were also present. In aqueous phase, Deltaproteobacteria (all eight genera) were present (Kryachko et al. 2012). This is due to favorable growth substrates like toluene and heptanes present in the aqueous phase. The microbial community was thriving in oil reservoir (MHGC oil field, southern Canada) originated from the source water (Folarin et al. 2013). Source water had a more diverse community than produced water. Injection water was dominated by *Clostridium*, Comamonadaceae, and *Flavobacterium*, while produced water was dominated by *Methanoculleus*, *Methanolinea*, and Methanosarcinales (Folarin et al. 2013).

Gao et al. (2014) studied the Lu (Sandstone, 37°C) and Liu (Conglomerate, 22.6°C) field blocks located in the Xinjiang oil field, in the Junggar Basin, Northwest China (Gao et al. 2014). Microbial communities of both reservoirs had aerobic bacteria, including *Sphingomonas*, *Azospirillum*, *Paracoccus*, *Ochrobactrum*, *Alcanivorax*, and Hydrogenophilaceae in injection wells. In reservoir strata, *Pseudomonas*, *Rhizobium*, *Arcobacter*, *Spirochaeta*, and *Bacteroides* were present.

Okoro et al. (2014) studied Obigbo (onshore, 30–40°C) and Bonga (offshore, 28–32°C) fields of Nigeria for the microbial communities involved in souring and corrosion (Okoro et al. 2014). SRB and NRB both were present in samples of Obigbo field. Corrosion scraps of both the fields had high number of SRB. Pyrosequencing of samples indicated anaerobic and methanogenic hydrocarbon degradation in all the samples of Obigbo field, while Bongo field had higher activity of SRB, NRB, and so-NRB. Corrosion-related experiment revealed that Bongo field had moderate general corrosion rates. In both oil fields, anaerobic, fermentative Porphyromonadaceae, nitrate-reducing Deferribacteraceae, Rhodobacteraceae, and *Marinobacterium* were present. In Obigbo field, methanogens were abundant mainly related to methylotrophic methanogen *Methanolobus*, other than *Methanosaeta* (convert acetate to methane and CO_2). Oil-degrading Firmicutes were also present in Obigbo oil field. Bonga oil field had marine bacteria *Parvibaculum* and *Thalassobaculum*. *Parvibaculum* is capable to degrade alkane and present in high fraction during presence of hydrocarbons in injection water. Rhodobacteraceae, Rhodospirillaceae, *Marinobacter*, and *Marinobacterium* dominated in produced water of Bongo field. Deltaproteobacteria, *Desulfovibrio*, *Desulfuromonas*, and *Geoalkalibacter* were also present in Bongo oil field (Okoro et al. 2014).

Gao et al. (2014) studied the dynamics of indigenous microorganisms from low-temperature oil of Xinjiang oil field located in Northwest China (Gao et al. 2015). Hydrocarbon oxidizing bacteria (HOB), NRB, and methane-producing bacterial abundance has increased after stimulation by adding nutrition (molasses and mineral salts), but not of SRM. *Pseudomonas*, *Ochrobactrum*, *Acinetobacter*, *Halomonas*, *Marinobacter*, *Methanoculleus*, *Methanosaeta*, *Methanocor pusculum*, and *Methanocalculus* increased during nutrient enrichment. All these organisms are involved either in bio-surfactant production or in gas production (methane and CO_2) and to MEOR (Gao et al. 2015). Recently another Canadian oil field production water (27°C) has shown the presence of *Ralstonia*, *Arcobacter*, *Sulfurospirillum*, *Methanohalophilus*, *Methanocalculus*, and *Methanolobus* sp. by 16S rRNA- and *cpn60*-based sequencing for microbial community analysis (Kryachko et al. 2017). The *cpn60* gene encodes the type I chaperonin protein. By targeting *cpn60*, finer discrimination among microbial taxa was achieved, which was shown by 16S rRNA gene targeting. The *cpn60* sequence-based phylogeny showed the presence of *Sulfurospirillum*, *Methanolobus*, *Pelobacter*, and *Bacillus* spp. in production water.

17.2.7 High-Temperature Reservoir Microbial Community

High-temperature reservoir communities have been studied by both culture-dependent (traditional) and culture-independent techniques (Table 17.2). Traditional culture-dependent microbial detection methods have limitation to cover all microorganisms. 16S rRNA gene cloning and sequencing overcomes this limitation. Analysis of 16S rRNA gene sequences of clones enables detection of almost complete biodiversity of the collected samples. Firstly, Orphan et al. (2000) combined the culture-dependent and culture-independent techniques for characterization of microorganisms from high-temperature oil field of California (Orphan et al. 2000). *Methanoculleus thermophilus*, *Methanobacterium thermoautotrophicum*, and *Methanothermococcus thermolithotrophicus* were detected. The parallel combination of both techniques is also used for investigating microbial diversity of Samotlor high-temperature oil field, Siberia, Russia. The observations of the culture-dependent technique showed the presence of Thermococcales and Methanobacteriales (Bonch-Osmolovskaya et al. 2003), and similar observations were recorded by the molecular approach (oligonucleotide microchip analysis). Several additional genera were also identified during hybridization experiments. *Desulfurococcus* sp. was also detected; generally its habitats are terrestrial hot springs and deep sea hydrothermal vents (Marteinsson et al. 2001). The analysis of 16S rRNA gene clone libraries revealed archaeal clones majorly of methanogens and sulfate reducers. Methanogens *Methanocalculus pumilus* (99% similarity) and *Methanocaldococcus infernus* (97% similarity) were also detected. Bødtker et al. (2008) studied the microbial community of the nitrate-amended North Sea oil fields (back-flowed injection water) (Bødtker et al. 2008). So-NRB genus *Arcobacter* and NRB *Terasakiella* were present; both are mesophilic in nature so that these bacteria are expected to grow at NIWR. Genus *Deferribacter*-related organism was also

Table 17.2 Microbial communities present in high-temperature oil fields

S. no.	Oil field names (high-temperature oil fields)	Bacterial communities	References
1.	California oil field	*Methanoculleus thermophilus*, *Methanobacterium thermoautotrophicum*, and *Methanothermococcus thermolithotrophicus*	Orphan et al. (2000)
2.	Samotlor oil field, Siberia, Russia	Thermococcales and Methanobacteriales, *Desulfurococcus*, *Methanocalculus pumilus,* and *Methanocaldococcus infernus*	Bonch-Osmolovsk-aya et al. (2003)
3.	North Sea oil field	*Arcobacter*, *Terasakiella*, *Deferribacter*, and *Sulfurimonas*	Bødtker et al. (2008)
4.	Alaskan Slope oil field, Canada	*Desulfomicrobium thermophiles*	Duncan et al. (2009)
5.	Huabei oil reservoir, China	*Pseudomonas* sp., *Serratia* sp., *Wolinella* sp. *Campylobacter* sp., *Thermoanaerobacter* sp., *Thermovenabulum* sp., *Thermotogales* sp., *Mycobacterium* sp., *Nitrospira*, *Thermodesulfovibrio* sp., and *Burkholderia* sp.	Li et al. (2006)
6.	Qinghuang oil field unit, China	*Thermoanaerobacter* sp., *Thermovenabulum* sp., *Geobacillus* sp., *Nitrospira* sp., *Thermodesulfovibrio* sp., *Fervidobacterium* sp. *Thermotogales* sp., *Sinorhizobium* sp. *Rhodocyclus* sp., *Comamonas* sp., *Hydrocarboniphaga* sp., *Sphingobacterium Methanobacterium*, *Methanothermobacter*, *Methanococcus*, *Methanobravibacter* sp., and *Nitrosopumilus*	Li et al. (2007)
7.	Daqing oil field, China	*Clostridium* sp., *Thauera* sp., *Hydrogenophaga* sp., *Pseudomonas* sp., *Eubacterium* sp., *Arcobacter* sp., *Desulfovibrio* sp., *Desulfovibrio profundus*, *Desulfuromonas*, *Desulfomicrobium*, *Desulfohalobium*, *Desulfonatronum*, *Desulfobulbus*, *Desulforhopalus*, *Desulfnema*, *Alcaligenes* sp., *Eubacterium* sp., and *Tepidiphilus* sp.	Wei et al. (2010)
8.	Dan and Halfdan oil field, North Sea	*Thermococcales*, *Archaeoglobus*, *Desulfacinum*, *Desulfomaculum*, *Methanothermococcales*, and *Desulfacinum*	Gittel et al. (2009)

(continued)

Table 17.2 (continued)

S. no.	Oil field names (high-temperature oil fields)	Bacterial communities	References
9.	Shengli oil field, China	*Methanothermobacter thermautotrophicus* and *Methanothermobacter wolfeii*	Cheng et al. (2011)
10.	Halfdan oil field, Denmark	*Deferribacter*, *Colwellia*, and *Methylophaga*	Gittel et al. (2012)
11.	Barrancas and Chihuido de la Salina (CHLS) oil field, Argentina	*Archaeoglobus*, *Thermococcus*, *Calditerrivibrio*, *Petrotoga*, and *Thiomicrospira*	Agrawal et al. (2014)
12.	Stolbovoye and Samotlor oil field, western Siberia	Methanosarcinales, Methanobacteriales, *Pelotomaculum* sp., and *Desulfotomaculum* sp.	Frank et al. (2016)
13.	Central Africa oil field	*Ochrobactrum* sp., *Halolactibacillus* sp., *Halococcus* sp., and *Haloplanus* sp.	Gales et al. (2016)
14.	Jiangsu oil field, China	*Thioalkalivibrio* sp., Alphaproteobacteria, *Methanomicrobia*, *Archaeoglobi*, *Thermococci*, and *Desulfotignum*	Li et al. (2016)
15.	Mexican oil field	*Desulfovibrio*, *Desulfomicrobium*, and *Desulfohalobium*	Zapata-Peñasco et al. (2016)
16.	Terra Nova oil field, Canada	*Archaeoglobus* sp., *Desulfotomaculum*, *Marinobacter*, and *Geobacillus*	Okpala et al. (2017)
17.	Western India oil field	*Tepidiphilus*, *Paenibacillaceae*, *Kocuria*, *Thauera*, *Thermoanaerobacte*r, *Thermovorax*, *Arthrobacter*, *Desulfotomaculum*, *Desulfomicrobium*, and *Thermodesulfobacterium*	Prajapat et al. (2018)

detected in nitrate-treated field. So-NRB of the genus *Sulfurimona*s was a major community when nitrate-treated sea water injection was performed (Bødtker et al. 2008). *Desulfomicrobium thermophilum*, isolated from Alaskan Slope oil fields (68°C), was the main SRB (Duncan et al. 2009).

Li et al. (2006) studied water-flooded high-temperature Huabei oil reservoir of China for the bacterial diversity by molecular analysis. The 16S rRNA gene clone libraries were constructed, and restriction fragment length polymorphism (RFLP) analysis was performed. For sequencing, a total of 337 clones were selected (74 OTUs) for final analysis. The results of sequencing and phylogenetic analysis showed that selected clones were related to Gammaproteobacteria (85.7%), Thermotogales (6.8%), Epsilonproteobacteria (2.4%), Gram-positive bacteria (2.1%; low G+C content), Gram-positive (<1%; high G+C content), Betaproteobacteria, and *Nitrospira* (each <1%). Both mesophilic (*Pseudomonas* like clones) and thermophilic bacteria were detected in these high-temperature oil

fields. RFLP of 337 clone libraries was analyzed; all clones were of 74 OTUs. Gammaproteobacteria (85.7% clones)-related bacteria *Pseudomonas* (80.7%) and *Serratia* sp. (4.5%) were abundant. The Epsilonproteobacteria (2.4% clone) related to *Wolinella* and *Campylobacter* were also abundant. Lower G + C Gram-positive bacteria related to *Thermoanaerobacter* sp. SL9 (97.6% identity), *Thermoanaerobacte*r sp. MET-G (96%), and *Thermovenabulum* sp. B8-otu12 (96.6% identity) sequences were detected. The Thermotogales (6.8% clones) related to *Thermotoga hypogea* SEBR 7054 identical sequence was detected. Other bacteria such as *Mycobacterium massiliense* CCUG 48898 (98.5% identity), *Nitrospira*, *Thermodesulfovibrio* sp. TGL-LS1 (96.6%; sulfate reducers), and *Burkholderia* sp. SAPII (100% identical) were also detected (Li et al. 2006).

Li et al. (2007) studied high-temperature, water-flooded petroleum reservoir of the Qinghuang Unit in China for microbial community analysis (Li et al. 2007). The bacterial (388 clones) and archaeal (220 clones) clone libraries were clustered with 60 and 28 phylotypes, respectively (Li, 2006 Molecular). Bacterial clones related to Firmicutes, Thermotogae, Nitrospirae, and Proteobacteria and archaeal clones related to methanogens (*Methanothermobacter*, *Methanobacter*, *Methanobrevibacter*, and *Methanococcus*) and *Thermoprotei* were present. The closely related species of Firmicutes were *Thermoanaerobacter keratinophilus* (99% similarity), *Thermoanaerobacter subterraneus* SL9 (99% similarity), *Thermovenabulum ferriorganovorum* (99% similarity), and *Geobacillus subterraneus* 34^T (98% similarity). The Nitrospirae-related species were *Nitrospira* sp. SRI-9 (96%), *Thermodesulfovibrio* sp. TGE-P1 (99%), *Fervidobacterium islandicum* AW-1 (99%), *Thermotoga* sp. KOL6 (99%), *Thermotoga thermarum* (99%), and *Thermotoga* sp. SRI-1 (97%). Proteobacterial sequences showed similarity with the closest cultivated species *Sinorhizobium* sp. R-25078 (98%), *Rhodocyclus* sp. HOD 5 (83), *Comamonas* sp. D22 (98%), *Hydrocarboniphaga* effuse AP102 (99%), *Sphingobacterium* sp. MG2. The archaeal sequences were related to subdivision Euryarchaeota; *Methanobacterium fomicicum* FCAM (99%), *Methanothermobacter thermautotrophicus* GC-1 (99%), *Methanobacterium subtrraneum* C2BIS (99%), *Methanococcus maripaludis* S2 (93%), *Methanobravibacter* sp. Mc30 (94%), and Crenarchaeota-related species was *Canditatus Nitrosopumilus maritimus* (82%).

Wei et al. (2010) studied SRB communities and their dynamics during the water-flooding process in Daqing Oil field (>45°C) (Wei et al. 2010). PCR-DGGE analysis showed that microbial communities were different in each treatment unit of oil field. Dominant microbial communities were related to *Clostridium* sp., *Thauera* sp., *Hydrogenophaga* sp., *Pseudomonas* sp., *Eubacterium* sp., and *Arcobacter* sp. SRB related to *Desulfovibrio* sp. and *Desulfovibrio profundus* were present in few numbers. They constructed the APS gene clone library and performed sequence analysis. APS analysis showed that Proteobacteria (*Desulfovibrio*, 65.2%) are the main species. Other than these, SRB genera *Desulfuromonas*, *Desulfomicrobium*, *Desulfohalobium*, *Desulfonatronum*, *Desulfobulbus*, *Desulforhopalus*, *Desulfnema*, and *Desulforhopalus* (34.8%) were also present. *Alcaligenes* sp. (99% identity), *Eubacterium* sp. (98%), and *Tepidiphilus* sp. (98%) were also detected by DGGE analysis of 16S rRNA gene (V3 region).

Gittel et al. (2009) compared the microbial communities of two adjacent high-temperature oil reservoirs (80°C), Dan and Halfdan oil fields of the North Sea (Gittel et al. 2009). Halfdan oil field was treated with nitrate to control the reservoir souring. They showed that prokaryotic community was dominated in reservoir in both the oil fields with lower diversity in Halfdan oil fields. Firmicutes (bacteria) and Thermococcales (Archaea) were dominant in both the fields. In nitrate-treated fields, exclusively Deferribacterales (nitrate reducers) were present. *Desulfacinum* and *Desulfomaculum* (SRB) were abundant in both the study sites. They concluded that *Archaeoglobus* was the main SRA that plays a role in hot reservoir souring. Nitrate treatment stimulates the nitrate reducers in the reservoir. Bacterial phyla Firmicutes; Gamma-, Delta-, and Epsilon-*proteobacteria*; and *Synergistes* were stimulated in Halfdan oil fields. In Dan PW, one clone affiliated to Alphaproteobacteria and Thermotogales was present. In Dan oil field, the diversity and species richness were higher than Halfdan oil fields. SRMs detected were related to genera *Desulfacinum* and *Desulfovibrio*, and very minor sequences were related to *Desulfomaculum geothermicum* (more abundant in Halfdan oil fields). The so-NRB was also detected in low numbers (high in Halfdan PW) and related to *Epsilonproteobacteria* from both oil fields' PWs. The analysis of Archaeal clone libraries revealed the phyla Thermococcales, Methanothermococcales, and Archaeoglobales. SRM *dsr*AB gene libraries suggested that *dsr*AB gene diversity and species richness were higher in Halfdan as compared to Dan. The clone library sequences were majorly related to *Archaeoglobus fulgidus*. Other SRMs were related to *Syntrophobacteraceae* (*Desulfacinum*) and *D. geothermicum*/*D. anilinii*. None of the sulfate reducers were related to genera *Anaerobaculum*, *Petrotoga*, and *Thermovirga*. These members are able to use thiosulfate or elemental sulfur for production of sulfide.

Syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis is an alternate pathway in high-temperature oil reservoirs. In this environment, the genus *Methanothermobacter*-related organisms were present (Gittel et al. 2009). Cheng et al. (2011) studied the Shengli oil field, China (Cheng et al. 2011). The analysis of 16S rRNA gene library showed the presence of *Methanothermobacter thermautotrophicus* and *Methanothermobacter wolfeii* VKM B-1829 (96.4% similarity)-related organisms in reservoir.

Gittel et al. (2012) reported microbial diversity and abundance in injection and production waters of high-temperature, nitrate-treated Halfdan oil fields of Denmark (Gittel et al. 2012). They quantified (16S rRNA and *nap*A gene) nitrate-reducing bacterial members of Epsilonproteobacteria and *Deferribacteres*. *Deferribacter* was the dominant (30%) microbial community in production fluids, but not in injection fluids (i.e., indigenous to the reservoir). Epsilonproteobacteria were present in low abundance in the production fluids. Gammaproteobacteria (genus *Colwellia*), Epsilonproteobacteria (<1%), and Gammaproteobacteria (methylotrophic genus *Methylophaga*) were present in this nitrate-treated oil fields (Gittel et al. 2012). Guan et al. (2013) studied four oil fields: Shengli oil field (63°C), Ba block of Huabei oil field (58 °C), Menggulin reservoir of Huabei oil field (37 °C), and Kelamayi oil field (21 °C) for the distribution and diversity of SRM by using 16S rRNA and *dsr*AB genes (Guan et al. 2013). In all four different temperature

reservoirs, *Desulfotomaculum* (higher in high-temperature reservoir) and *Desulfobacter* (high in mesothermic reservoir) were dominant, while *Desulfovibrio*, *Desulfobulbus*, and *Desulfomicrobium* were absent in all four oil fields. With decrease in temperature of these reservoirs (63–21°C), SRM communities increased. *Desulfotomaculum* presence was correlated with environmental variables and species distribution (temperature, depth, and presence of acetate, propionate, and sulfate), while *Desulfomicrobium*, *Desulfobacter*, and *Desulfobulbus* abundance is correlated with sulfur and salinity. Salinity and concentration of acetate increase the *Desulfobacterium* distribution in reservoir fluids (Guan et al. 2013).

The Barrancas field (80°C) and the Chihuido de la Salina (CHLS) field, Argentina (65–70°C), were flooded with high sulfate-containing injection water (Agrawal et al. 2014). In produced water, *Archaeoglobus*, *Thermococcus*, *Calditerrivibrio*, and *Petrotoga* were abundant. In a water plant of Barrancas field, the presence of *Thiomicrospira* (85%, so-NRB, Gammaproteobacteria) led to reoxidation of sulfide into sulfate (Agrawal et al. 2014).

Frank et al. (2016) studied the microbial communities of fluids from wells in pristine and water-injected, high-temperature Stolbovoye (non-water flooded) and Samotlor oil field (performed PWRI) of western Siberia (Frank et al. 2016). They analyzed microbial communities by PCR-DGGE and sequencing approach. The Stolbovoye oil field had more diversity than the Samotlor oil field. The Firmicutes were the common phylotypes present in both the oil fields. Methanosarcinales and Methanobacteriales were only detected in Samotlor oil field. In Stolbovoye samples, archaeal amplification was not attained, while Samotlor fields had *Methanosaeta thermophila* (89–98% similarity)-related organism revealed by DGGE analysis of 16S rRNA gene. The DGGE analysis of *dsr*B gene fragments revealed the presence of *Pelotomaculum propionicum*- and *Desulfotomaculum* sp.-related organisms in Samotlor samples, while in Stolbovoye oil sludge contained *Desulfotomaculum acetoxidans* DSM 771-related organisms.

Gales et al. (2016) studied the indigenous microbiota of the preserved sand core of hypersaline oil reservoir (reservoir temperature 43°C) located in Central Africa (Gales et al. 2016). The 16S rRNA gene sequencing showed the abundance of *Ochrobactrum* sp. (99% similarity), *Halolactibacillus halophilus* (98% similarity), *Halolactibacillus miuriensis* (98% similarity), *Halococcus hamelinensis* (99% similarity), and *Haloplanus natans* (98% similarity) (Gales et al. 2016).

The pyrosequencing of geographically distinct petroleum reservoir of China (22 oil reservoirs) showed that different microbial communities inhabit in these different reservoirs (Gao et al. 2016). The main factors that cause variation in whole microbial communities of reservoirs are the mining pattern, spatial isolation, reservoir temperature, salinity, and pH of fluids. Most of the oil fields share the similar dominant microbial communities. Bacterial population was more heterogeneous within and among the reservoirs compared to the archaeal population. The sequencing data showed the abundance of the rare microbial organisms from these underground reservoirs. The Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Thermotogae, and Synergistetes are dominant bacterial phyla (average 93.63%) in every oil field, while dominated archaea are related to *Euryarchaeota*

(average 97.86%) (Gao et al. 2016). Hu et al. (2016) studied Ivishak formation (83–83°C), Kuparuk formation (65–80°C), and Schrader Bluff formation (25–27°C) located in Alaska North slope oil field (Hu et al. 2016). They used genome-resolved approach for studying the microbial community distribution and detailed metabolic prediction. Microbial community richness increased with decrease in temperature (60–40°C). Clostridiales were abundant in Schrader Bluff oil reservoir. Ivishak formation was dominated by *Thermoanaerobacter*, *Desulfonauticus* sp., and *Archaeoglobus fulgidus* (99.46% sequence similarity). Kuparuk formation was dominated by *Archaeoglobus fulgidus* and *Thermodesulfobacterium commune*. The high-temperature Jiangsu oil field of China had Gammaproteobacteria (*Thioalkalivibrio*-related species) and Alphaproteobacteria (Li et al. 2016). In samples, archaea like Archaeoglobi and Thermococci were dominant. The *Desulfotignum* sp. was the dominant SRM present in the samples. The mesophilic Desulfovibrionales SRB was common in produced waters (Zapata-Peñasco et al. 2016). The *Desulfovibrio*, *Desulfomicrobium*, and *Desulfohalobium* were predominantly present in connate water (fossil water) of Mexican offshore, non-waterflooded and high-temperature reservoirs (Zapata-Peñasco et al. 2016). Hyperthermophilic sulfate-reducing archaea *Archaeoglobus* spp. were present in Terra Nova production waters (Okpala et al. 2017). The *Desulfotomaculum* (50–70°C enrichment), *Marinobacter* (30–50°C enrichment), and *Geobacillus* (40–65°C enrichment) were also isolated from various high-temperature oil field enrichments. Thermophilic NRB (*Geobacillus* and *Marinobacter*) reduce nitrate to nitrite only (not into N_2), so that this produced nitrite helps in nitrite-mediated inhibition of SRM. Prajapat et al. (2018) isolated various microbes from western Indian oil fields which grew on either sulfate- or nitrate-amended media containing electron acceptors and donors (Prajapat et al. 2018). *Tepidiphilus*, *Paenibacillaceae*, *Kocuria*, *Thauera*, *Thermoanaerobacte*r, *Thermovorax*, and *Arthrobacter* were isolated on nitrate-amended media, while *Desulfotomaculum*, *Desulfomicrobium*, and *Thermodesulfobacterium* were isolated on sulfate-amended media (Prajapat et al. 2018).

17.3 Oil Sands

Oil sands are unconventional oil deposits that contain sand, clay, water, and petroleum (bitumen). These are found in many countries; among them Canadian oil sands are the largest. Other than Canada, Kazakhstan, Russia, and Venezuela (non-bituminous) have large quantities of oil sands.

Canada has extremely large quantities of natural bitumen deposits and world's third largest oil reserves and fossil fuels distributed into three different regions in Alberta: Athabasca, Cold Lake, and Peace River (Government of Alberta 2010). Athabasca oil sands represent significant oil resource, are rich in crude bitumen, and constitute about 170 billion barrels of proven bitumen reserve (Kasperski and Mikula 2011). Approximately 20% of this resource is present within 75 m of the surface and allows extraction by mining or by in situ methods (Ramos-Padrón et al.

2011). Only 3% of the total reserve (4800 km^2) can be surface mined as the bitumen is available very near to the surface. To date, approximately 715 km^2 of this area has been exploited for its resources (Chalaturnyk et al. 2002; Government of Alberta 2010).

During extraction of bitumen from oil sands ore using water-based techniques, the mined ores create large volumes of mineral suspensions composed of process-affected water, sands, silt, clay, and residual bitumen, leading to the formation of tailing ponds (Chalaturnyk et al. 2002; Kasperski and Mikula 2011). Chemically tailing ponds are complex mixtures of compounds (including organic acids and unrecovered hydrocarbons) that are detrimental for the environment. Therefore, management of tailing ponds is an important part of oil sand operations. Various processes have been developed for the management of tailing ponds, e.g., treatment with lime [$Ca(OH)_2$] and CO_2 and thickening using suitable thickeners (Chalaturnyk et al. 2002).

In the current practice, approximately 80% of water that is released in the tailing ponds is recycled back for the extraction process (Ramos-Padrón et al. 2011). Although the presence of mineral suspensions in the water forms a stable network of fines making densification process slow, several new treatment technologies have been developed with an aim to accelerate the densification process. Typically, gypsum ($CaSO_4.2H_2O$) is added to tailings to increase the rate of densification and promote remediation of tailing ponds. The addition of calcium sulfate as a tailing flocculating agent favors the SRB metabolism, consequently the production of sulfide (Chalaturnyk et al. 2002). Due to which the population of methanogens gets increased and consequently methane production enhanced leading to a concern in tailing ponds (Fedorak et al. 2002). Tailing ponds have diverse microbial communities (An et al. 2012) which perform sulfur and one carbon (C1) cycle metabolism as the main microbial activities (Ramos-Padrón et al. 2011). The microbiota of tailings has not yet been studied adequately, which is critical in estimating the extent of benefits and risks involved in microbial catalyzed processes (i.e., H_2S and methane gas production) in tailing ponds. *Desulfocapsa* spp./ *Thiobacillus* spp. may be the key players in sulfur metabolism while *Methanosaeta* spp./*Methanolinea* spp. are the leading methanogens (Ramos-Padrón et al. 2011). It was also seen that at some depths, there are “pockets” of sulfate that are not being reduced by SRB, probably due to the lack of biodegradable electron donors or may be another group of bacteria like SOB were potentially oxidizing sulfide to sulfate. One approach to check microbes which are taking the lead in these processes is to target specific genes that participate in the sulfur oxidoreduction and methane production pathways for quantitative real-time PCR (qPCR). qPCR can be very helpful to accurately estimate the distribution of microbial communities participating in these mechanisms (Agrawal and Lal 2009).

Oil sands tailing ponds have different microbial communities in various upper to lower layers (Table 17.3). Anaerobic deeper layer was found to be dominated by *Pelotomaculum*, *Syntrophus*, *Smithella* spp. (syntrophs), *Desulfocapsa*, and *Desulfovibrio* spp. (SRB). At 16.8 m depth, *Actinobacteria* are the main community, while at greater depths, methylotrophic genera *Methyloversatilis*, *Azospirillum* (N_2 fixer), and *Gemmata* are present. The *Rhodococcus* and *Pseudomonas* sp. (Y2) were

Table 17.3 Microbial communities present in oil sands/oil sands tailing ponds

S. no.	Oil sands/oil sand tailing pond name	Bacterial communities	References
1.	Athabasca oil sands tailing pond	*Desulfocapsa* sp., *Thiobacillus Methanosaeta* sp., *Methanolinea Pelotomaculum*, *Syntrophus*, *Smithella* sp., *Desulfocapsa*, *Desulfuovibrio Actinobacteria*, *Methyloversatilis*, *Azospirillum*, and Gemmata	Ramos-Padrón et al. (2011)
2.	Northern Alberta oil sands tailing pond	*Rhodococcus* and *Pseudomonas* sp. (Y2)	Demeter et al. (2014)
3.	Muskeg River mine oil sands	Methanomicrobiales and Epsilonproteobacteria	Hubert et al. (2012)
4.	Athabasca river oil sands	Chloroflexi, *Rhodoferax*, *Thiobacillus*, *Smithella*, *Methanoculleus*, and *Methanolinea*	Yergeau et al. (2012)

also isolated from oil sand process water samples from a northern Alberta oil sands tailing pond (Demeter et al. 2014). In the formation water of Muskeg River mine, Archaea related to Methanomicrobiales (CO_2 reducer) and bacteria related to Epsilonproteobacteria were present (Hubert et al. 2012). Microbial communities in the Athabasca River, its tributaries, and tailing pond sediments were analyzed by next-generation sequencing (Yergeau et al. 2012). Classes Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Bacteroidetes, Firmicutes, and Chloroflexi were present in all collected samples. *Rhodoferax*, *Thiobacillus*, *Smithella,* and Archaeal communities (*Methanoculleus* and *Methanolinea*) were present only in sediments (Yergeau et al. 2012).

17.4 Coal Bed Methane

Coal bed methane (CBM) is a natural gas extracted from absorbed solid matrix of coal. This methane is different from conventional methane because CBM is stored or absorbed within the coal. Composition of CBM is methane and trace amounts of ethane, nitrogen, carbon dioxide, and few other gases. Composition of CBM mainly depends on properties of coal. CBM reservoirs are mostly distributed in Australia, United States, United Kingdom, Canada, Kazakhstan, and India. Deep CBM reservoirs are formed due to thermal coalification processes, whereas shallow CBM reservoirs are formed due to in situ microbial activity (Barnhart et al. 2016; Faiz and Hendry 2006; Ritter et al. 2015; Strąpoć et al. 2011).

The microbiology of CBM is related to fermentative, acetogenic, and methanogenic bacteria (Table 17.4). In CBM, initially fermentative bacteria hydrolyze the fermentable complex to produce acetate, long-chain fatty acids, CO_2, H_2, NH_4^+, and HS^-. Produced H_2 and CO_2 are used by acetogenic bacteria to produce acetate. The acetogenic bacteria can demethoxylate low molecular weight ligneous materials and aromatic compounds to acetate (McInerery and Bryant 1981). The produced H_2, CO_2, and acetate are utilized by methanogens to produce methane.

Table 17.4 Microbial communities present in coal bed methane (CBM)

S. no.	CBM field name	Bacterial communities	References
1.	Shikari coal field, Hokkaido, Japan	*Methanoculleus*, *Methanolobus*, *Acetobacterium*, and *Syntrophus*	Shimizu et al. (2007)
2.	Powder River Basin of Wyoming, USA	*Acidaminobacter* sp., *Clostridium* sp. *Syntrophomonas* sp., *Mollicutes* sp., *Spirochaeta* sp., *Desulfomicrobium* sp., *Aeribacillus*, Actinobacteria, *Methanosarcinales*, *Methanocaldococcus*, *Methanothermococcus*, *Methanobrevibacter*, *Methanobacterium*, and *Methanomicrobium*	Barnhart et al. (2016), Green et al. (2008) and Klein et al. (2008)
3.	Australian CBM	Desulfuromonaceae, Geobacteraceae, Desulfovibrionaceae, Comamonadaceae, *Shigella*, *Escherichia*, *Halomonadaceae*, *Clostridiaceae*, *Veillonellaceae* *Actinobacteria*, *Archaeoglobus*, and *Methanobacterium* sp.,	Li et al. (2008), Midgley et al. (2010), Shimizu et al. (2007) and Vick et al. (2018)
4.	Canadian CBM	Alphaproteobacteria, Firmicutes, Bacteroidetes, and Spirochaetae, *Methanocorpusculum*	Penner et al. (2010)
5.	USA CBM (Texas)	*Pseudomonas*, Veillonellaceae, *Methanosarcina barkeri*, *Geobacter*, and *Methanosaeta concilii*	Jones et al. (2010)
6.	China CBM	*Methanolobus*, *Hydrogenophaga* *Arcobacter*, and *Azonexus*	Guo et al. (2012)
7.	Indian CBM	*Methanobacterium*, *Methanothermobacter*, *Methanoline*a, *Azonexus*, *Azospira*, *Dechloromonas*, *Thauera*, and *Pseudomonas stutzeri*-BHU	Singh et al. (2012) and Singh and Tripathi (2013)

Generally, methanogenic bacteria present in consortium and interspecies H_2 transfer are common (Zinder 1993).

Concept of microbially enhanced CBM (MECoM) is similar to microbial enhanced oil recovery concept introduced by Scott (Scott 1999). By the application of bacterial consortia and nutrients, it is possible to produce new methane from coal, and also reservoir permeability can be increased by the utilization of coal, waxes, and paraffin. Indigenous microbes alone can perform this job (Baker and Herson 1994; Cookson 1995). Various laboratory trails showed that bacterial consortia are able to convert coal into methane (Volkwein et al. 1994). The common methanogens

that thrive in CBM are *Methanosarcina*, *Methanolobus*, *Methanobacteria*, *Methanocorpusculum*, *Methanosaeta*, *Methanococci*, *Methanoculleus*, and *Methanoregula*. The bacterial community present in CBM is related to Firmicutes, Spirochaetae, Bacteroidetes, and Proteobacteria (Strąpoć et al. 2011).

Molecular characterization of microbial communities of Shikari coal field, Hokkaido, Japan, was studied by Shimizu et al. (2007). The results of 16S rRNA gene sequencing showed that hydrogenotrophic and methylotrophic methanogens are present in groundwater samples. The hydrogenotrophic archaea related to genus *Methanoculleus* and the methylotrophs related to genus *Methanolobus* were dominant in methanogen clones. Bacterial clones were related to *Acetobacterium* and *Syntrophus*, and these have symbiotic association with methanogens (Shimizu et al. 2007). Phylogenetic analysis of wellbore water samples of Powder River Basin of Wyoming, USA, had Methanosarcinales (acetoclastic methanogen)-related OTUs (Green et al. 2008). In the produced water of Powder River Basin CBM, *Methanocaldococcus*, *Methanothermococcus*, *Methanobrevibacter*, *Methanobacterium*, and *Methanomicrobium* were the dominant methanogens, while in coal *Methanobacterium* and *Methanothermococcus* were the dominant methanogens (Klein et al. 2008). Bacterial communities related to Firmicutes, Spirochaetae, and Proteobacteria were present in Powder River Basin CBM samples (Green et al. 2008). Firmicutes related to *Acidaminobacter hydrogenoformans* (obligate anaerobes, acetogen), *Clostridium* spp. (anaerobic cellulolytic bacteria), *Syntrophomonas* (secondary fermenters, acetate producer), and Class *Mollicutes* (cultured species *Acholeplasma*, facultative anaerobes, ferment carbohydrates) were present. *Spirochaetae* related to *Spirochaeta* spp. Buddy (obligate/facultative anaerobic and fermentative acetate producers) and Proteobacteria related to Deltaproteobacteria class (*Desulfomicrobium* sp.) were present. Pyrotag analysis of Powder River Basin samples showed that *Aeribacillus* (in sandstone cores) and Actinobacteria (in claytone or sandstone cores) were the dominant microbes (Barnhart et al. 2016).

The analysis of CBM water and coal samples collected from eastern Australia showed dominance of Gram-negative bacteria, whereas archaea and Gram-positive bacteria were low in number (Li et al. 2008). Coal samples had exclusively archaeal (genus *Archaeoglobus*) and Alphaproteobacterial communities, while water samples had Beta-, Gamma-, Delta-, and Epsilon-proteobacteria and Bacteroidetes (Li et al. 2008). However, another study showed the presence of *Methanobacterium* species (reduce CO_2 to methane) in Gippsland Basin of southeast Victoria, Australia (Midgley et al. 2010). Li and coworkers had not uncovered these species, which could be due to randomly chosen clones or bias in amplification by the used primer set. In Australian coal formation, bacterial diversity is vast, mainly composed of Firmicutes, Proteobacteria, and Actinobacteria (Li et al. 2008; Midgley et al. 2010). Coal samples had Deltaproteobacteria, families like Desulfuromonaceae, Geobacteraceae, and Desulfovibrionaceae (Midgley et al. 2010). The Geobacteraceae (use ferric iron as terminal electron acceptor) was also found in earlier studies (Li et al. 2008; Shimizu et al. 2007). The family Desulfovibrionaceae (*Desulfovibrio* sp.) is of sulfate reducers; sometimes they perform fermentation and

produce H_2. The Betaproteobacteria of the family Comamonadaceae was also found in CBM. The Gammaproteobacteria of families Enterobacteriaceae (genera *Shigella* and *Escherichia*), Halomonadaceae (fermentative), and Xanthomonadaceae and Firmicutes families Bacillaceae1, Clostridiaceae, and Veillonellaceae were commonly found in Australian, Japan, Canadian, and US coal formation (Green et al. 2008; Li et al. 2008; Midgley et al. 2010). Actinobacteria were present in low abundance in these studies (Li et al. 2008). Vick et al. (2018) studied the microbial profiling of formation water of Bowen, Sydney, and Surat CBM basins of Australia (Vick et al. 2018). The Bowen basin had higher average level of biodiversity than other two basins. All these basins were dominated by Proteobacteria, Firmicutes, Euryarchaeota, and Bacteroidetes (Vick et al. 2018).

The *Methanosarcina* spp. were the dominant methanogens in CBM reservoirs of Alberta, Canada (Penner et al. 2010). Another CBM, Illinois Basin located in Canada had *Methanocorpusculum* dominant genus in production fluids. H_2-utilizing methanogenesis is the terminal process present in this CBM. Alphaproteobacteria, Firmicutes, Bacteroidetes, and Spirochaetae bacterial clades (13% from total clones) and remaining clones related to archaea (mainly genus *Methanocorpusculum*) were present in Canadian coal bed.

Jones et al. (2010) used two approaches for the stimulation of methane from nonproduction coal collected from test well drilled in south Texas (Jones et al. 2010). They have used the biostimulation (native population was stimulated by adding nutrition) and bioaugmentation (addition of nutrition and bacterial consortium) approaches. In these approaches, bioaugmentation approach generated more methane than biostimulation. In biostimulation, *Pseudomonas*, Veillonellaceae, and *Methanosarcina barkeri* were enriched, while in bioaugmentation, *Geobacter* and *Methanosaeta concilii* were enriched.

Most of the earlier studies of microbial communities focused on CBM were based on conventional gene cloning and sequencing methods. Guo et al. (2012) used 454 pyrosequencing method for better understanding of microbial diversity in CBM (Guo et al. 2012). They collected various samples (coal, rock, and produced water) from Liulin district, a part of the Lishi-Liulin nose structure of Eastern Ordos Basin in China. The methanogen *Methanolobus* was dominant in all the collected samples, and this archaeon was able to utilize the methyl/methanol for methane production. This pathway is rarely reported compared to predominantly reported acetotrophic and hydrogenotrophic pathways (Strąpoć et al. 2010). *Methanolobus* was also found in CBM located in Japan and Alaska (Shimizu et al. 2007; Strąpoć et al. 2010). Strapoc et al. (2010) suggested that methylotrophic methanogenesis pathway is the efficient pathway for the CBM generation in Alaska (Strąpoć et al. 2010). The *Hydrogenophaga* (H_2-utilizing bacteria), *Arcobacter* (role in nitrogen cycle), and *Azonexus* (denitrifying bacteria) were detected in both coal and rock samples.

An Indian coal bed formation water (Jharia, India) analysis showed the presence of methanogens *Methanobacterium*, *Methanothermobacter*, and *Methanoline*a and bacteria *Azonexus*, *Azospira*, *Dechloromonas*, and *Thauera* (Singh et al. 2012). These microbial communities have a role in hydrogenotrophic methanogenesis, nitrogen fixation, nitrate reduction, and polyaromatic compound degradation.

Singh and Tripathi (2013) isolated the *Pseudomonas stutzeri*-BHU (99% identity) from Jharia coal bed, India. This strain produces the biosurfactant rhamnolipids in the presence of lignite coal (Singh and Tripathi 2013). The produced biosurfactant is useful in coal dust control, enhancement of gas permeability in coal bed, and removal of coal ash.

17.5 Potential Applications and Future Perspectives

Souring Prevention

Much of the world's oil is produced by secondary recovery where the injection of water is done to maintain reservoir pressure and sweep the oil to the producing wells. In this process, the fraction of produced water increases and oil proportion decreases with time. Produced water separated from crude oil is again mixed with makeup water drawn from a suitable source and reinjected in the reservoir. This is known as produced water reinjection (PWRI). PWRI is generally done for onshore oil fields, while seawater is injected offshore introducing 28 mM sulfate and causing sulfide production by SRB (souring). PWRI also enhances souring if the sulfate is present in makeup water or in situ in the reservoir.

Souring control can be done by including nitrate into seawater/PWRI injection water in the fields where resident temperature is above 60°C. Nitrate injection induces growth of hNRB and sulfide-oxidizing NRB (so-NRB). The former compete for the same oil organics as the SRB, whereas the latter oxidize sulfide directly. Most so-NRB are chemolithotrophs using CO_2 as the sole carbon source. SRB are also strongly inhibited by nitrite, produced by NRB.

In the high-temperature oil fields, bulk microbial activity (SRB) occurs at NIWR, since the reservoir temperature is too high for microbial activity. NIWR needs to be treated with nitrate for control of souring. In the high-temperature fields, growth of NRB is expected downstream from the nitrate injection point till the near-injection wellbore region (NIWR). Analysis of the back-flowed injection water revealed the presence of sulfide-oxidizing NRB of the genus *Arcobacter* and heterotrophic NRB of the genus *Terasakiella*. These microbes are mesophiles, and growth is only possible at NIWR in high-temperature oil field. NIWRs are generally cooler than the rest of the reservoir due to continuous flow of cold sea water or PWRI.

On the other hand, souring control in low temperature requires good planning, time, and effort. This is because of establishment of discrete zones of hNRB activity in the NIWR and of SRB activity deeper in the reservoir. MHGC oil fields of Canada are an example of souring control by nitrate injection which has a low resident temperature (30°C) and is injected with water with a low sulfate concentration. Since the start of nitrate injection in 2007, the sulfide formation declines by 70% in the first 5–7 weeks, but this was followed by recovery to pre-nitrate injection levels. Establishment of separate zones of hNRB activity in the NIWR and of SRB activity deeper in the reservoir was hypothesized to cause the recovery. Under these conditions nitrate injection needs to be done for longer period with high nitrate pulse (7 years

for MHGC oil fields) before the souring control may be achieved. Souring control can be indicated by nitrate and nitrite breakthrough in producing wells.

Microbially Enhanced Oil Recovery (MEOR)
Various tertiary recovery technologies have been adopted by oil companies due to increasing water cuts. MEOR is one of these technologies in which bacteria are used either native or introduced to produce more oil. MEOR can improve oil production by decreasing oil viscosity/interfacial tension or by blocking permeable zones (conformance control).

MEOR technologies are developed in such a way that initially bacterial or MEOR agents are injected into the reservoir, followed by incubation of the reservoir for the bacteria to grow, followed by reinjection of the water for additional oil recovery.

A number of laboratory and field studies have been conducted for MEOR. Injection of bacteria is often coupled with injection of carbohydrate (molasses) as a carbon and energy source. Carbohydrate fermentation typically yields 90% fermentation products (acids, alcohols, gases, biosurfactants) and only 10% biomass, so that oil properties can be improved in favor of EOR. However, this is not always successful as many times ex situ bacteria, when injected into the reservoir, do not behave in the similar way as in laboratory. Another strategy can be using the native reservoir population of the bacteria for exopolysaccharides, gases, surfactant, and production of other MEOR agents. This can be done by injection of molasses, other fermentation products, biopolymers, hydrocarbons, and/or nitrate. This will not only produce large amount of gases, surfactants, etc. but also produce biomass that can plug the high permeable zones. This will result in change in the path of water that will come in contact with oil earlier not assessable. Recently research projects for application of biopolymers that can be used for polymer flooding and also MEOR agents are being tried in petroleum laboratories.

Successful application of MEOR technologies requires successful alteration and modification of microbial communities in the reservoir that can produce fermentation products and biomass in the existing reservoir situation. Application of more than one EOR technology in combination that can overcome the drawbacks of each other will be the future for EOR technologies, and beyond any doubt, MEOR is going to play a significant role in this future synergism. Making choices of MEOR requires rigorous understanding of both petroleum engineering and microbiology. Probably in the near future, oil companies will be looking for the personnel having these sets of skills.

17.6 Conclusions

It is now well established fact that microbial communities found in any hydrocarbon resource environment are not only responsible for generation of oil but also plays important role in oil production. For the number of the isolates, the optimal growth conditions correlate well with the in situ oil reservoir conditions and indicate that these isolates are the part of a complex microbial ecosystem with an active

biogeochemical cycling of carbon and other nutrients. Souring in the oil field recycles the sulfate present in the reservoir. This is done by a number of SRB species with a large diversity of physiological properties. Advanced sequencing technologies revealed that methanogenic archaeal populations represent a significant part of the microbial community in petroleum reservoirs. In a number of petroleum reservoirs, methanogenic conditions prevail in the absence of electron acceptor such as sulfate and linked with biogenic methane production. In addition to SRB and methanogens, fermentative bacteria with different metabolic capabilities are recovered from oil reservoirs. Fermentative bacteria grow on a wide range of substrates, including carbohydrates and/or proteins and produce a variety of volatile fatty acids with acetate being the most common end product formed. Although much attention has been focused in the last three decades on the microbiology of petroleum reservoirs, our knowledge of the biogeochemical cycles in the reservoirs is limited. Hence, identification and detection of microbes in oil reservoirs continue to be a promising area of research in petroleum microbiology.

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Mining Human Microbiome for Therapeutics

18

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Abstract

Human microbiome is known to play a key role in human health along with the host genetic makeup and environmental factors. Research studies carried out in the past decade have now established a clear association of human microbiome with various metabolic and gastrointestinal diseases through the state of dysbiosis. Extensive efforts have been made to develop novel and effective methods such as the use of probiotics and fecal microbiota transplantation (FMT) as corrective measures to improve the human health. It is indeed now important to understand the concepts and developmental process of such alternative biotherapeutics to develop potential treatment regimens. This chapter gives insights into the history, development, and the current scenario of the use of these techniques. The process of how these techniques have been developed through mining of the human microbiome is discussed broadly.

Keywords

Human microbiome · Probiotics · Prebiotics · Fecal microbiota transplant

18.1 Introduction

Human microbiome has been recently recognized as the "last human organ under active research" (Baquero and Nombela 2012). This organ is fundamentally a complex assemblage of trillions of microbes majorly comprising bacteria, archaea, and viruses, which outnumber the number of human body cells (Baquero and Nombela 2012; Cénit et al. 2014). Although the proportion of microbial cells to

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body cells is still a debatable aspect, microbiome research has now moved from taxonomic identification of the human-associated microbes to bioengineering of these complex communities to improve human health (Kali 2015). Extensive efforts have been put up in exploring and understanding the microbial communities residing in and on our body (Odamaki et al. 2016; Ottman et al. 2012). Advancements in DNA sequencing technology and computational methods in handling the "Big data" over the past decade have enabled researchers to dive deeper into the functions of these tiny creatures. The then "least understood organ of the human body" (Human Microbiome) is now being studied through novel integrated approaches to obtain deeper insights into the genetic potential, especially of the gut bacteria, to drive the important body functions such as digestion, metabolism, and host immunity (Baquero and Nombela 2012; Odamaki et al. 2016).

It is now a well-established fact that the human gut bacteria are known to play a key role in maintaining the body homeostasis and the healthy state. Any disruptions in the microbial community structure, termed "dysbiosis," are associated with several gastrointestinal and metabolic diseases, ranging from inflammatory bowel disease (IBD), ulcerative colitis (UC), and type 2 diabetes (T2D) to other autoimmune disorders such as celiac disease (Bassols et al. 2016; Zheng et al. 2017). These disruptions are attributed to multiple factors such as changes in diet and lifestyle and overuse of antibiotics (Odamaki et al. 2016). Considering the rapid changes in dietary habits and lifestyle, especially in the Western population, these diseases are now considered as one of the biggest threats to human health. These studies have now clearly shifted the previous paradigm of considering the factors of "genetic makeup" and "environmental conditions" as the only factors influencing human health; microbiome is now an additional and equally important factor that is known to drive the human health (Baquero and Nombela 2012; Cénit et al. 2014; Kali 2015; Odamaki et al. 2016).

Research studies have now been directed toward exploring novel and potential approaches to correct the dysbiotic state to normal. Understanding the pathophysiology of the disease is indeed a prerequisite to design and develop any treatment. Thus, researchers across the globe have explored the microbiome of several populations in both healthy and diseased states to understand the differences in microbiome profile in both these conditions. It is now evident from these reports that, firstly, there exists a substantial difference between healthy and diseased subjects and, secondly, there exists a significant difference in the microbiome structure of different populations (Bhute et al. 2016; Kumbhare et al. 2017). Thus these studies opened up new opportunities for researchers to explore new therapeutic measures/agents in the human microbiome. It thus became an important aspect to mine the human microbiome to find potential leads to develop corrective measures to modulate the microbiome and thus eventually improve the human health (Cénit et al. 2014; Kali 2015). There has been a constant interest in the use of probiotics in various gastrointestinal diseases due to the health benefits imparted by these beneficial microbes. The concept of probiotics which was traditionally restricted to few bacterial strains has now been widened up from multi-strain formulations to its combinatorial use with prebiotics (Glanville et al. 2015; Hill et al. 2014)

Furthermore, a novel technique, namely, fecal microbiota transplantation (FMT), has emerged as a promising therapy in certain gut-associated diseases (Ahmad and Akbar 2016; Allegretti et al. 2017; de Groot et al. 2017; Moayyedi et al. 2015a; Smits et al. 2013). Although used traditionally in various parts of the world, this technique is now newly understood through microbiome perspective and holds a substantial potential to be used as a corrective measure in the treatment regimens designed for patients suffering from metabolic and/or gastrointestinal diseases or even for gastric infections. It is thus important to understand the evolution of both these important measures, i.e., probiotics and FMT, and their current implementations in improving human health. This chapter will collectively focus on the development of probiotics and FMT, from the mining of human microbiome, as measures to improve the human health through microbial modulations and microbial interventions.

18.2 Probiotics as Microbiota Modulators

18.2.1 Probiotics: A Story that Began a Long Time Ago

The era of probiotics began in the twentieth century, with Elie Metchnikoff who correlated the longevity of Bulgarian peasants to their high consumption of fermented milk (Anukam and Reid 2007; Mackowiak 2013). This observation of fermented milk and its bacteria on health led him to suggest that *all microbes were not dangerous for health* and that it could be beneficial to replace the disturbed flora to normal by enrichment of specific bacteria, such as the use of lactic acid bacteria (LAB) to regain the normal gut health (Anukam and Reid 2007; Mackowiak 2013). Concurrently, a French pediatrician, Henry Tissier, observed that infants suffering with diarrhea had very few Y-shaped gram-positive bacteria in their feces, while these were observed dominantly in healthy infants' feces (Ventura et al. 2015), which were later identified as *Bifidobacterium.* Although this concept of beneficial bacteria was forgotten during the era of antibiotics and vaccines, recent studies on the commensal microbiota have renewed interest in their contribution (Aminov 2010; Penesyan et al. 2015). The concept of probiotic therapy took a major footstep toward reality in 1930. The Japanese microbiologist Minoru Shirota fist discovered bacterium that survived passage through the gut after ingestion. He subsequently isolated and cultivated this bacteria which is now known as *Lactobacillus casei* strain Shirota (A. C. Brown and Valiere 2006; Gogineni 2013). This became the first bacteria-containing drink to be commercially marketed as Yakult in 1935—a product that continues to be sold worldwide today (Gogineni 2013).

18.2.2 Definition of Probiotics

The word "probiotic" has undergone several different denotations over the past decades and is now being used as an umbrella term. Therefore it is important to

understand how the definition of probiotics has evolved over time. Lilley and Stillwell in 1962 described probiotics as “substances secreted by one microorganism and stimulated the growth of another microorganism” (Tannock 2004). It was only in 1971 that Sperti reused the term “probiotics” and described probiotics as “tissue extracts which stimulated microbial growth”(Tannock 2004). The first use of this word to describe a microbial food supplement was by Parker in 1974, and he defined probiotics as “organisms and substances which contribute to intestinal microbial balance” (Tannock 2004). Later, in 1989, Fuller modified the definition of probiotics as “live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Tannock 2004). In this modified definition, the word “substances” was removed which includes antibiotics. It also stressed the viable nature of the preparations. It is now evident that, in order to be effective, microorganisms should be viable (Fuller et al. 1992).

Later advancements in our understanding of probiotics indicate that along with the attributes described previously, these beneficial bacteria should embrace not only effects on the intestinal flora but also on other groups, such as those inhabiting the vagina and respiratory tract (Tannock 2004). Havenaar and Huis isn’t Veld (1992) made an attempt to extend the definition and proposed the definition as “a mono or mixed culture of live microorganisms which, applied to animal or man, affect the host beneficially by improving the properties of the indigenous microflora” (Tannock 2004). Two years later, even this definition was not adequate. For example, should one also include microbial stimulants such as the bifidogenic factors and also include effects obtained in plants and even in food? Taking this into consideration, the definition was modified to “a probiotic is a preparation consisting of live microorganisms or microbial stimulants which affects the indigenous microflora of the recipient animal, plant or food in a beneficial way” (Lee et al. 2008). All these definitions refer to microorganisms and would include bacteria, yeasts, fungi, viruses, and bacteriophages. All these groups of organisms except the animal and plant viruses have been shown to have beneficial effects when administered to humans. The microorganisms included as probiotics should be nonpathogenic and components of the normal microflora, e.g., lactic acid bacteria and some nonpathogenic variants of pathogenic species can function in the same way as traditional probiotics (Amara and Shibl 2015; Fijan 2014; Ayichew et al. 2017). For example, *Enterococcus faecium* and *Enterococcus faecalis* can also protect against infection by the respective virulent parent strain (Vikas C Ghattargi et al. 2018a, b). Thus “probiotics” was becoming increasingly difficult to define, while some scientists prefer to call them “microbial feed supplements.” At present, the vast understanding of probiotics includes the yoghurt (M. Bernardeau and Vernoux 2013). The latest definition stated by World Health Organization (WHO) in 2002 is “probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host,” and this in now widely accepted definition all over the world (Ozen and Dinleyici 2015).

18.2.3 Sources of Probiotic Strains

It's a known fact that dairy and dairy-related products are a good source of probiotics (Sornplang and Piyadeatsoontorn 2016). Lactic acid bacteria (LAB) and *Bifidobacterium* obtained from fermented milk have been used as probiotics for centuries. The traditional spontaneous fermented milk contains complex compositions of LAB species and therefore provides a useful source of probiotic strains. A total of 148 LAB strains were isolated from *Kurut*, the traditional naturally fermented yak milk from China in which *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophiles* were found to be predominant among the microbial population (Sun et al. 2010). Also, yeasts and *Lactobacillus* strains with probiotic properties have been isolated from Masai milk, koumiss (a fermented milk drink), and kefir grains, and these microorganisms are able to influence immune responses (Cassanego et al. 2017; John and Deeseenthum 2015; Prado et al. 2015; Schutte 2013). Studies conducted recently to evaluate traditional fermented products as potential natural sources of probiotic bacteria revealed that most of the microorganisms isolated from fermented products belong to the genus *Lactobacillus* (Bernardeau et al. 2006; Cunha et al. 2013; Florou-Paneri et al. 2013; Tamang et al. 2016). Remarkably, in a recent work, a *Weissella* strain was isolated from Nigerian fermented foods and selected for its probiotic potential, and now, many reports state its inherent probiotic capacity (Abriouel et al. 2015; Anandharaj et al. 2015; Elavarasi 2014; Leite et al. 2015). Cheese is a dairy product with potential source of probiotic microorganisms, and *L. plantarum* strains have been isolated from Italian, Argentinean, and Bulgarian cheeses (Abriouel et al. 2015; Anandharaj et al. 2015; Elavarasi 2014; Leite et al. 2015).

Other studies show that probiotic strains are also found in nondairy fermented foods (Sornplang and Piyadeatsoontorn 2016). In vitro experiments have demonstrated that certain bacterial strains, isolated from meat (*L. sakei, L. curvatus* and *Staphylococcus carnosus*) and fruits (*L. paracasei* and *L. plantarum*), can display functional and metabolic properties similar to those of human intestinal bacteria (Arief et al. 2016; Fontana et al. 2013; Zhang and Cai 2014). In addition, a recent work described the isolation of a *Lactobacillus* strain from brines of naturally fermented Alorena green table olives. Moreover, *L. buchneri* P2, isolated from pickled juice, demonstrated probiotic properties, such as cholesterol reduction, acid and bile tolerance, and antimicrobial activity (Zeng et al. 2010).

Interestingly, breast milk, which was previously considered to be sterile, has now shown to be an important source of beneficial microbes for infants' health (Bouchachia and Bessam 2016; Gavin and Ar 1977). The lactobacilli present in human milk are genotypically different from those isolated from the skin, and the LAB strains present in breast milk were also observed in the faces of the

corresponding infants (Bouchachia and Bessam 2016; Fernández et al. 2013; Kozak et al. 2015; Martín et al. 2009; Murphy et al. 2017; Rodriguez 2014; Singh et al. 2017). Only recently it has been accepted that breast milk constitutes an interesting source of the probiotic LAB and *Bifidobacterium* (Fernández et al. 2013). In addition, it has been reported that breastfed infants have fewer allergies and gastro-intestinal infections than formula-fed infants; therefore, the intestinal microbiota in the breastfed infant might be considered to be ideally healthy (Murphy et al. 2017). Human breast milk predominantly comprises staphylococci, streptococci, micrococci, lactobacilli, enterococci, lactococci, and bifidobacteria (Bouchachia and Bessam 2016; Fernández et al. 2013; Gavin and Ar 1977; Kozak et al. 2015; Martín et al. 2009; Murphy et al. 2017; Rodriguez 2014; Singh et al. 2017), and its intake favors the predominance of *Bifidobacterium* and lactobacilli in the infant intestinal microbiota. Several authors have reported that lactobacilli isolated from breast milk are an efficient alternative to the use of commonly prescribed antibiotics for the treatment of infectious mastitis during lactation (Arroyo et al. 2010; Hurtado et al. 2017; Jiménez et al. 2008). Moreover, it was reported that two *Lactobacillus* strains isolated from human breast milk enhanced natural and acquired immune responses through the activation of the natural killer and T-cell subsets and the expansion of regulatory T cells (Pérez-Cano et al. 2010).

Human gastrointestinal tract (GIT) is known to be an important source of probiotics, and more than 500 different bacterial species reside in the adult human gut (Rajilić-Stojanović and de Vos 2014). In fact, many of the probiotic strains used today have been isolated from this source, such as *L. gasseri and L. reuteri* (Fontana et al. 2013). In addition, it has been reported that *L. fermentum*, isolated from human colonic mucosal biopsy samples, possesses antimicrobial activities against food-borne pathogens (Varma et al. 2010). A common factor that is must for probiotics is they must always colonize the intestinal tract to exert their effects. In fact, certain probiotics, e.g., *B. longum*, reside in the human intestinal microbiota, but others (e.g., *L. casei* and *B. animalis*) do not (Derrien and van Hylckama Vlieg 2015; Health 2017; Soccol et al. 2010). Most of the probiotic strains, such as *B. longum* and *L. acidophilus* RY2, were isolated from the fecal samples of healthy adults and infants, respectively. Notably, in concordance with breast milk, several studies have reported the isolation of probiotics from breastfed infant faces (Derrien and van Hylckama Vlieg 2015; El-Tayeby et al. 2017; Fontana et al. 2014; Soccol et al. 2010).

18.2.4 Characterization of Functional Aspects of Probiotic Cultures

Performing characterization of the probiotic properties of the strains before their use on a commercial scale is necessary (Fijan 2014). One must provide proof of efficacy for each strain and define the different positive health benefits of the probiotic strains. Characterization of probiotic properties can be carried out using various in vitro tests for functional, probiotic aspects and safety assessment (Jose et al. 2015; Shewale et al. 2014). Several criteria have been used for the selection of probiotic strains, of which the most commonly employed are the survival under the stressful

GIT conditions (low pH and high bile salt concentrations); the ability to transitory colonize the GIT, which is related with the adhesion to mucus and/or intestinal epithelium; and the antimicrobial activity through the production of antimicrobial molecules or the ability to inhibit/displace the adhesion of pathogens (Fijan 2014; Jose et al. 2015; Shewale et al. 2014). These attributes are described in detail in the following sections:

18.2.4.1 Resistance to Gastric Conditions (Acid and Bile)

An important attribute for probiotic bacteria functions is survival and growth in the intestinal tract, i.e., intestinal stressful conditions. After ingestion, the probiotic bacteria must survive the passage through the GIT and reach the colon in order to exert their beneficial effect. The low pH in the stomach and the high concentration of bile salts in the small intestine, which act as biological detergents disrupting the cell membrane, are the principal challenges that probiotics must overcome. Once they survive in the GI tract, probiotic bacteria have the opportunity to influence the populations and activities of different intestinal bacteria. Therefore, demonstrating that a probiotic can survive GI transit and influence GI tract flora is important for establishing probiotic characteristics (Hassanzadazar et al. 2012; Ruiz et al. 2013).

18.2.4.2 Adherence to Mucus and/or Human Epithelial Cells

Adherence of probiotics to intestinal epithelium is known to be a prerequisite for colonization of the gastrointestinal tract and has long been considered one of the most important selection criteria for probiotic microorganisms. Adhesion to the intestinal mucosa may prevent probiotic cells from being washed out; and therefore, enabling temporary colonization, immune modulation, and competitive exclusion of pathogens. In general, it is assumed that probiotic strains are able to inhibit the attachment of pathogenic bacteria by means of steric hindrance at enterocyte pathogen receptors. Adherence of bacterial cells is usually related to cell surface characteristics, and hence, it is important to determine bacterial properties such as cell surface hydrophobicity and the adhesion ability of the probiotic cultures. Characteristics of probiotic strain to serve as an effective prophylactic agent include avid adherence to epithelial cells, interference with the adherence of other bacteria, production of bacteriocins, and production of H_2O_2 capable of inhibiting the growth of pathogens (Alemka et al. 2010; Bernet et al. 1993; Vélez et al. 2007).

18.2.4.3 Auto-aggregation and Co-aggregation Assays

In order to manifest beneficial effects, probiotic bacteria need to achieve an adequate mass through aggregation. Consequently, the ability of probiotics to aggregate is a desirable property. The cellular aggregation helps not only in the transient colonization but also in providing a protective shield to the host system due to formation of a bacterial biofilm over the host tissue. It also enhances the gastrointestinal persistence of the organism in vivo, as well as its adhesion to epithelial cells in vitro. Organisms with the ability to co-aggregate with other bacteria such as pathogens may have an advantage over non-co-aggregating organisms, which are more easily removed from the intestinal environment. Studies on the mechanism of auto-aggregation have

shown the presence of gene (*agg*) and the protein located on the cell surface. Many authors have reported that the co-aggregation abilities of probiotic species might enable them to form a barrier that prevents colonization by pathogenic bacteria, and this feature may constitute a protective mechanism against infection (Armas et al. 2017; Tareb et al. 2013).

18.2.4.4 Antimicrobial Activity Against Potential Pathogens

Antimicrobial activity is thought to be an important means for probiotic bacteria to competitively exclude or inhibit activities of harmful or pathogenic intestinal microbes. These inhibitory substances are basically various by-products of cells' metabolism that have antagonistic effect pathogens. These compounds are classified as biosurfactants that inhibit adhesion; the organic acids, bacteriocins, and hydrogen peroxide inhibit growth; and the co-aggregation molecules block the spread of the pathogens. Antimicrobial compounds produced by probiotic bacteria include organic acids (lactic and acetic acid), hydrogen peroxide (in environments in which oxygen is present), diacetyl, β-hydroxypropionaldehyde, or bactericidal or bacteriostatic peptides and proteins (Prabhurajeshwar and Chandrakanth 2017; Šušković et al. 2010).

18.2.4.5 Hypocholesterolemic Activity

The reduction of total cholesterol or low-density lipoproteins found in human plasma is thought to lower the risk of coronary heart disease. Probiotic cultures have been suggested to take up cholesterol in the presence of bile and in the absence of oxygen, both conditions present in the intestinal tract thus playing a role in reduction of these blood lipids. The most abundant bile salts in humans are cholate, chenodeoxycholate, and deoxycholate, which are normally conjugated with either glycine (75%) or taurine (25%). Bile salts are water-soluble end products of cholesterol and are synthesized in the liver. The deconjugation of bile salts leads to decreased solubility and hence lower reabsorption in the enterohepatic system, thereby resulting in an increased demand for cholesterol as a precursor of bile salts. A functional Bile salt hydrolase (BSH) enzyme could help in the depletion of cholesterol in the host, resulting in protection against cardiovascular diseases. Since cholesterol assimilation is reported to be strain-dependent, evaluation for reduction in cholesterol needs to be studied for each strain (S. J. Kim et al. 2017b; Kumar et al. 2012; Pereira et al. 2003).

18.2.4.6 Antioxidative Activity

The interest in reactive oxygen species (**ROS**) or free radicals in biology and medicine is evident because of their strong relationship with phenomena such as aging and because they are a major cause of many degenerative diseases, such as atherosclerosis, cancer, cardiovascular diseases, inflammatory bowel diseases, old age dementia, and arthritis. ROS comprises not only oxygen-centered radicals such as $O_2^{\bullet-}$ and OH but also nonradical derivatives of oxygen such as H_2O_2 and hypochlorous acid. It is well known that free radicals and other ROS are continuously being produced in living organisms. The most common systems are

superoxide dismutase and high internal concentrations of Mn^{2+}. Certain research groups also reported that some species of lactobacilli produced a heme-dependent catalase, which can degrade H_2O_2 at a very high rate, blocking the formation of peroxyl radicals. The ability of lactic acid bacteria to create low oxidation-reduction potential needed for their optimum growth probably is related to some of these systems (Mishra et al. 2015; Wang et al. 2017). The above described assays will help in finding the true potential of an organism to be a probiotic candidate. Apart from these assays one could also target various gut diseases and metabolic disorders.

18.2.5 Mechanisms of Action

The mechanisms of action of probiotics are not well understood in humans, however, are documented through in vitro and animal model studies with the consequent limitation to extrapolate these results to human beings (M. Bernardeau and Vernoux 2013). Therefore, the European Food Safety Authority (EFSA) has a prejudice toward the worldwide accepted definition of probiotics defined by WHO and thus also the health claims of marketed probiotics because of the lack of sufficient proof.

The mode of action of probiotics may be related to the modulation of the host's gut microbiota (Bermudez-Brito et al. 2012; Dimidi et al. 2017). Probiotics exert the resistance for colonization of pathogenic bacteria, thereby limiting their colonization, and this is called as barrier effect (Mennigen and Bruewer 2009). The pathogenic bacterial inhibition may be due to the production of broad-spectrum bacteriocins, short-chain fatty acids (decrease of the pH), and biosurfactants with an antimicrobial activity and also due to competition for binding sites in the gut (Dimidi et al. 2017; Walker 2008). Thus, the adherence of probiotic bacteria and inhibition of pathogenic bacteria helps in the improvement of the gut mucosa. Thus the barrier function helps in the quality of tight junctions between intestinal epithelial cells as these probiotics act at the level of signaling by increasing the mucus layer and production of defensins, as well as by improving their physiological barrier function (Bermudez-Brito et al. 2012; Walker 2008).

Probiotics can affect the immune system by various components, viz., the metabolites and its cell wall constituents (Dimidi et al. 2017). Most of the immune cells are located in the gut and especially in the small bowel, and thus these probiotics or their products are familiar to host cells as they are well equipped with recognition receptors. Hence, gut epithelial and gut-associated immune cells are the main targets (Reid 2016; Walker 2008). It has been proved that probiotics adhere and relate soluble factors to the host gut epithelial cells and trigger a signaling cascade leading to immune modulation. The internalization of probiotic bacteria by Dendritic cells (DCs) which are localized below the epithelial cells leads to further consequences. They lead to the activation of regulatory T cells and differentiation of T helper lymphocytes (Th), inducing the production of pro- or anti-inflammatory cytokines. Bacteria with probiotic potential, especially lactic bacteria, may have different effects, depending on the cytokine profile (Bermudez-Brito et al. 2012; Dimidi et al. 2017; Reid 2016; Walker 2008). The effects may be local and limited to stimulation of gut

immunity (e.g., stimulation of secretory IgA production) or systemic. Gram-positive bacteria have teichoic acids in the cell walls that are involved in the anti-inflammatory activity; this was probably due to involvement of Toll-like receptor 2 (TLR-2)-dependent pathway. Thus probiotics are able to maintain the integrity of the mucosal gut barrier against the invasive action of enteropathogenic *Escherichia coli* in a TLR-independent way (Bischoff et al. 2014; Bron et al. 2017; Yu et al. 2015).

18.2.6 Modulatory Effects in Diseases

Probiotics exhibit direct effects in the GIT and indirect effects in other parts of the body (Markowiak and Slizewska 2017). These effects are due to the impact that probiotics have on immunity via changes in inflammatory mediators. As probiotics break down nutrients, they increase the building of proteins, vitamins, and functional compounds in the body by increasing absorption and digestibility of nutrients (La Fata et al. 2018; Vieira et al. 2013). Symptoms treated with probiotics include diarrhea, gastroenteritis, irritable bowel syndrome, inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis, cancer, depressed immune function, food allergies, infant allergies, depression, hyperlipidemia, and liver disease, and a few of them are discussed below (Daliri and Lee 2015; Parvez et al. 2006).

18.2.6.1 Diarrhea

Diarrhea is a multicausal disease and thus is known by various names, namely, travellers' diarrhea, antibiotic-associated diarrhea (ADD), *C. difficile* diarrhea, and rotavirus diarrhea. Antibiotic-associated diarrhea (AAD) is a common complication caused due to use and abuse of antibiotic. The frequency of AAD can be high (26–60%) during outbreaks and endemic periods (McFarland et al. 2016). Risk factors for AAD include broad-spectrum antibiotics, and host factors include age, health status, gender, and exposure to nosocomial pathogens (Jabbar and Wright 2003; Riddle et al. 2016). AAD typically occurs after 2–8 weeks of exposure to antibiotics as a result of disrupted intestinal microflora. Intestinal microflora acts as a protective barrier against the colonization of intestinal pathogens. The disturbance caused due to excessive use of antibiotics, thus without this protective barrier, makes these patients susceptible to infection by opportunistic pathogens (Jump 2013). There is a lack of therapies for the treatment of AAD, and current methods include discontinuation and restriction of the high dose of antibiotics. In such cases, probiotics can reverse the gut dysbiosis condition by reestablishing the disrupted intestinal microflora. This in turn enhances the immune responses, ultimately clearing pathogens and toxins from the GIT (L. V. McFarland 2006). Thus, the use of probiotics (single species or multispecies) has shown its effectiveness in the prevention of AAD (Videlock and Cremonini 2012). There has been comprehensive meta-analysis that inspects the preventive role of probiotics in ADD in different settings, such as different age groups and probiotic strains. The results were not consistent as there was a large variation in the dosage of probiotics, formulations used, frequency, timing of administration, and other various factors (Sazawal et al. 2006). Consideration of all these factors is a must when interpreting and extrapolating the results.

Despite variation in methods and intervention among the various studies carried across world, the overall analysis suggests that probiotics are efficacious in preventing acute diarrhea by at least 21%. This analysis also compared the efficacy of probiotics in preventing acute diarrhea among children and in adults. Although a statistically significant protection was observed in both children and adults, the effect size was significantly higher among children when compared with adults. These differences may be due to differences in colonization and gut flora in children and adults (Issa and Moucari 2014; McFarland 2006; Sazawal et al. 2006; Videlock and Cremonini 2012).

Clostridium difficile-associated diarrhea (CDAD) is an another form of diarrhea and usually affects as high as 30% of the diarrhea cases (Starr 2005; Wistrom 2001). *Clostridium difficile* is a spore-forming, gram-positive bacterium described in 1935 in newborn infants (Ghose 2013). Continuous and cumulative exposure to antibiotics like quinolone is a definite risk factor for CDAD (Ghose 2013; Starr 2005; Wistrom 2001). Antibiotics like fluoroquinolones, cephalosporins, macrolides, clindamycin, and carbapenems are among the high-risk groups for the causal of CDAD immediately within 1 week (Brown et al. 2013; Büchler et al. 2014). So, CDAD is considered as a severe form of AAD, and it seems clinically relevant to evaluate probiotics for the prevention. Single- and multicomponent probiotics have been used in the treatment of CDAD, and very few have been successful (Hickson 2011; Varankovich et al. 2015). A meta-analysis published on the use of probiotic have shown best successful in CDAD in by the use of *Saccharomyces cerevisiae* reducing the disease by 18% (Hickson 2011; Varankovich et al. 2015). Drawing conclusions from these publications has been difficult due to selection bias, small numbers, and heterogeneity of patients, types, or dosage of probiotics.

In conclusion, the available literature provides sufficient proof of the usefulness of probiotics in the prevention of various forms of diarrhea, and various species have been used and the variation by genera has been seen. However, there is a need for sufficient evidence, and trials should be carried out to improve the implementation of probiotics in such severe stages of diseases.

18.2.6.2 Irritable Bowel Syndrome (IBS)

IBS is a common chronic gastrointestinal disorder characterized by abdominal pain, bowel dysfunction, and bloating (Bharucha et al. 2017; Camilleri and Choi 1997). About 15% of the population is affected at some point during their lifetime, in the West (Card et al. 2014). IBS has been classified by their predominant bowel habit as follows: diarrhea-predominant IBS, constipation-predominant IBS, and IBS with alternating bowel movements; the available drug therapies for the treatment of IBS are poor (Canavan et al. 2015; Costanian 2015). Studies on gut microbiome have shown gut dysbiosis in the IBS; thus, probiotics can be an effective solution (Distrutti et al. 2016).

Many clinical studies investigated the therapeutic benefits of probiotics in IBS (Mazurak et al. 2015; Pélerin and Desreumaux 2016). The use of multispecies probiotics has shown favorable effects in controlling symptoms of IBS. Two trials with combination of multispecies probiotics for 5 months have shown that

symptoms like abdominal pain and flatulence became significantly lower in the probiotics group (Scaldaferri et al. 2013). The content of the probiotic was *L. rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve*, and *Propionibacterium freudenreichii* species *shermanii* JS (Lyra et al. 2010). Published meta-analyses for the use of probiotics in IBS have shown to reduce the IBS symptoms globally (Derwa et al. 2017; Ghosh 2004; Sheil et al. 2007). There has been heterogeneity overall in the analysis done in these meta-analyses due to various factors, viz., species or strains of the microorganisms used, treatment, and duration. Moreover, the interindividual gut microflora of the enrolled patients plays an important role in introducing the variation in results (Loh and Blaut 2012).

While using probiotics for the treatment of IBD, one should be definitive of the strain or spp., dosage, duration, and effectiveness of a probiotic. One should now look at the well-designed large-scale clinical trials that will help in proper diagnostic solution. This will also help to deepen our understanding about the mechanism of probiotics and also provide insights into the pathophysiology of IBS.

18.2.6.3 Inflammatory Bowel Diseases (IBDs)

IBD is a group of chronic inflammatory disorders of the gastrointestinal tract—mostly represented by Crohn's disease (CD) and ulcerative colitis (UC) (Loh and Blaut 2012). Many factors are thought to be the cause of IBD, viz., genetic, immunologic, and environmental factors, and the disease is most prevalent in Western countries. Moreover, it has become more widespread in societies of all age (Matricon et al. 2010). Recent studies have highlighted the role of gut microbiota in triggering, maintaining, and exacerbating the symptoms of IBD (Rubin et al. 2012). Specific microbes are found to be overrepresented in IBD, i.e., state of dysbiosis. Thus, probiotics could help in restoration of gut microbiome from the dysbiosis state and probably can cure IBD (Dalal and Chang 2014; Rubin et al. 2012). Few studies on the efficacy of probiotics in CD have been demonstrated with small number of patients. A longer remission and improved intestinal barrier permeability were seen in CD for *Saccharomyces boulardii* (Vieira et al. 2013). A Cochrane review and a meta-analysis (Derwa et al. 2017; Ghosh 2004) of randomized placebo-controlled clinical trials have stated the inefficiency of *Saccharomyces boulardii*; therefore, use of probiotics for preventing recurrence in CD is not well suited.

In ulcerative colitis, the efficacy of VSL#3 (*Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus bulgaricus*, and *Streptococcus thermophilus*) was proved in several research studies including in children (Bibiloni et al. 2005; Tursi et al. 2010). These studies provide proof of efficacy in the maintenance and remission, in patients with moderate UC only, and thus further studies are necessary (Bibiloni et al. 2005; Tursi et al. 2010). VSL#3 is approved for the prevention and the maintenance of remission of UC, and the efficacy is stated also in referral European guidelines (Tursi et al. 2010). But, further studies are essential to characterize specific mechanisms of actions in IBD. Since probiotics are becoming an appropriate therapeutic option, it is important to determine the potential of strains that possess the highest efficacy.

18.2.6.4 Celiac Disease

Celiac disease (CD) is a chronic autoimmune enteropathy caused by gluten intake and is now very common in all the age groups (de Sousa Moraes et al. 2014). Current therapy for celiac disease is compulsory and whole exclusion of gluten from dietary sources (Tavakkoli and Green 2013). However, these therapies have many drawbacks on practical grounds, for example, having a complete gluten-free diet (GFD). Thus, the need for other promising therapeutic is envisaged. Solutions such as tissue transglutaminase inhibitors, genetically modified gluten, and zonulin inhibitors have been around as recent solutions; however, use of probiotics can be a potential alternative methodology (Bakshi et al. 2012; de Sousa Moraes et al. 2014). As with other conditions, dysbiosis is very common in CD, so probiotics may help to reverse the gut conditions while improving the gut lining.

The reduced levels of bifidobacteria and lactobacilli in CD patients have been noted in various cases (de Sousa Moraes et al. 2014). Thus, supplementing these probiotic bacteria could be the best way to revert the dysbiotic state of gut microbiome. Reports suggest that probiotic spp. have been found to digest gluten and also alter gluten polypeptides in some cases. The best example is of probiotic preparation VSL#3, a multispecies content, which was found to lower the toxic properties of wheat flour through the extended fermentation (de Sousa Moraes et al. 2014; Saez-Lara et al. 2015). The hydrolysis of gliadin was observed due to the presence of enzymes digesting proline-rich peptides and aminopeptidases. This was not the case when comparison was made to other commercial probiotic products: Oxadrop, Florisia, and Yovis. Furthermore, the spp. of VSL#3 were individually tested to degrade gliadin, and no single sp. was self-sufficient in degrading gliadin peptides (Martinello et al. 2017; Saez-Lara et al. 2015). Thus, they must be used together with other strains to exert the beneficial effect against celiac disease (Angelis et al. 2006). The VSL#3 may prove to be more effective along with gluten-free diet in the treatment of celiac disease (Angelis et al. 2006).

The use of multispecies and single content has to be evaluated as there is a lack of consensus regarding the shifts in bacterial composition after the intake of probiotics. Future studies should be focused on microbiota characterization with benefits to gut health, with special emphasis on strains capable of producing anti-inflammatory effects along with gluten degradation. Additionally, it is indeed very important to conduct studies including a larger sample size to reach any substantial conclusion.

18.2.6.5 Necrotizing Enterocolitis (NEC)

Necrotizing enterocolitis typically occurs in premature births, during the third week of life. This has been attributed to damage to the intestinal tract due to loss of mucosal thickness, where microbes grow and cause necrosis and perforation. NEC affects up to 10% of infants, weighing less than 1500 g, with mortality rates being more than 50% depending on severity. The gut microbiome studies of preterm versus term infants have shown dysbiosis, thus a factor predisposition to NEC (Sanders et al. 2013). The dysbiosis in microbiome may be due to the high prevalence of antibiotic usage in these premature infants. The immature intestine in

preterm infants is especially prone to inflammation and loss of epithelial integrity and thus difficult to diagnose (Nanthakumar et al. 2000).

Probiotics have potential to improve the epithelial integrity and thus can reduce the progression (Bron et al. 2017). Thus, probiotics have been tested clinically for NEC. Probiotic studies involving strains and species of *Lactobacillus, Bifidobacterium*, *S. thermophiles*, and *Saccharomyces* have shown the reduction in frequency and mortality. Nevertheless, heat-killed preparations of *L rhamnosus* GG significantly reduced the incidence of NEC (Nanthakumar et al. 2000). The American Academy of Pediatrics recognizes probiotics as preventive measures for NEC in case of very-low-birth-weight infants. However, additional studies are necessary to identify the dose and strain of probiotic before issuing clinical recommendations (Sanders et al. 2013). Few strains such as *Bifidobacterium animalis* CNCM I-3446 have been shown to be effective toward prevention of NEC and not as an effective treatment regime.

In conclusion, there has been a growing evidence to support the potential use of probiotics in the prevention and treatment of various human diseases. One should understand that only some and/or specific organisms may be effective in certain disease manifestations. Further, one should establish proper guidelines for their specific use along with the efforts to demonstrate underlying mechanisms of action. Carefully selected and fully tested probiotic strains will probably provide alternatives for individuals in whom conventional medical therapies have failed to promote health, and perhaps, in the future, they may serve as a first-line choice of therapy for some patients.

18.2.7 Psychobiotics

Psychobiotics are "living organisms, upon sufficient ingestion produce health benefits to patients with psychiatric illnesses" (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). Thus, the term "psychobiotics" can be considered as a novel class of probiotics having applications in psychiatric medicine (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). Commensal gut microbes *Lactobacillus* spp., *Bifidobacterium* spp., and *Saccharomyces* spp. are known to exert several beneficial health benefits as shown in earlier diseases (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). Some of the intestinal microbes such as *Lactobacillus acidophilus*, *L. casei*, *Bifidobacterium infantis*, *B. longum*, and spp. of genera *Escherichia*, *Bacillus*, *Saccharomyces*, *Streptococcus*, and *Enterococcus* do produce neurotransmitters, viz., serotonin, norepinephrine, catecholamines, acetylcholine, and gamma-aminobutyric acid (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). These neurotransmitters modulate the expression of neurochemical receptors and thus act on the way between brain-gut axis, resulting in psychotropic effects (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). These effects are mainly antidepressant and anxiolytic.

The mechanisms of action of psychobiotics are not yet clearly understood as compared to those of probiotics (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). There have been studies that provide mechanistic insights; however, these studies are carried out in rodents, and they possibly suggest the role between microbiome and the brain. This gut-brain axis includes bacteria-enteric nervous system interactions, vagal signaling, short-chain fatty acids, gut hormones, and bacteria-derived blood metabolites (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). The vagus nerves and enteric nervous system connect the intestine with the central nervous system. Major and important metabolic products of gut microbial activity are the short-chain fatty acids (SCFAs), viz., butyrate, propionate, and acetate (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). The functioning of psychobiotics can also be related to the SCFAs exerting effects through G-protein-coupled receptors. The other possible mechanism can be attributed to hypothalamic-pituitary-adrenal (HPA) axis (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). This axis involves the brain and the adrenal glands, and it is involved during various stress responses. The system becomes dysfunctional during chronic stress or illness. This hypothesis probably indicates the key role played by microbes in causing mood disorders in gut-related diseases (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016).

Supplementation of *Bifidobacterium* has shown to provide protection against peroxidation of lipids and decreasing the brain monoamine oxidase activity, potentially increasing inter-synaptic neurotransmitter levels (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). In a randomized double-blind clinical trial, probiotic mixture containing *L. helveticus* and *B. longum* was given for a 30-day period and showed less psychological distress as compared to the placebo control (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). These studies provided clear evidence of microbiota and brain connection. Microbiome studies on psychiatric disorders, viz., hepatic encephalopathy and autism-spectrum disorder (ASD), have been carried out (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). Healthy male and female participants ($n = 40$) were administered a placebo product or a mixture of several probiotic species for over a period of 4 weeks. These spp. were *Bifidobacterium bifidum* W23, *B. lactis* W52, *Lactobacillus acidophilus*, *L. brevis*, *L. casei*, *L. salivarius*, and *L. lactis* (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). In comparison to the placebo group, probiotic-treated participants exhibited substantially reduced reactivity to sad mood as assessed by Leiden Index of Depression Sensitivity Scale (Kali 2016; Misra and Mohanty 2017; Sarkar et al. 2016). Similarly, in another double-blind trial, having probiotic in milk drink for 3 weeks showed significant improvement in mood.

The above studies have shed some light on the future prospects of psychobiotics. However, the evidence in human population is still limited. Further clinical studies are recommended to provide stronger evidence in favor of psychobiotics as an affordable, adaptable, and more compliant mode of treatment for mental illness.

18.2.8 Prebiotics

Prebiotics are oligosaccharides from food origins that are not digestible by the host but provide selective stimulation for growth and activity of specific members of the gut microbiota (Vieira et al. 2013). The most well-known prebiotics are inulin, lactulose, fructooligosaccharides (FOS), and galactooligosaccharides (GOS) (Umu et al. 2017). These prebiotics promote the growth of beneficial bacteria in GIT and subsequently promote homeostasis and good health (Umu et al. 2017).

Carbohydrates such as fiber, viz., pectin, gums, beta-glucan, cellulose, and lignin, are not digested in the upper gastrointestinal tract (Slavin 2013; Umu et al. 2017). This is attributed to the absence of enzymes to degrade these carbohydrates. In fact, these substances are selectively fermented by resident bacteria in the colon into SCFAs, viz., acetate, propionate, butyrate, and lactate. The role of these SCFAs is well known for their positive benefits in maintaining gut health, thus making dietary fibers potential prebiotics (Druart et al. 2014; Liu et al. 2015; Nicolucci and Reimer 2017; Slavin 2013; Umu et al. 2017; Vieira et al. 2013). These can be effective solutions for maintaining healthy gut and also improving the mucosal immune system (Druart et al. 2014; Liu et al. 2015; Nicolucci and Reimer 2017; Slavin 2013; Umu et al. 2017; Vieira et al. 2013).

Oligosaccharides, namely, inulin and oligofructose, have been studied as prebiotics and have shown to increase fecal bifidobacteria significantly even at low levels of consumption (5–8 g per day). A very-long-chain inulin from *Cynara scolymus* was found to have a pronounced prebiotic effect in human subjects (Calame et al. 2018; Elli et al. 2008; Francisco 2012). The ability to favorably alter the intestinal microflora has been demonstrated by a number of other fiber, plant, fruits, and vegetable sources. The comparison of insulin and gum from acacia was shown to produce a greater increase in bifidobacteria and lactobacilli for gum than inulin. Further, gastrointestinal side effects, viz., gas and bloating, were reduced (Calame et al. 2018; Elli et al. 2008; Francisco 2012). Also, dextrins from wheat have shown to increase lactobacilli and bifidobacteria and reduce *Clostridium perfringens* (Calame et al. 2018; Elli et al. 2008; Francisco 2012). "Psyllium" is a fiber made from the husks of the *Plantago ovata* plant's seeds. It forms a bulk-forming laxative and was found to have prebiotic potential in a small ($n = 11$) study in women (Calame et al. 2018; Elli et al. 2008; Francisco 2012).

In another study, a prebiotic effect was observed over the consumption of whole grains (wheat, maize) and bananas every day. The intake of whole grains and banana mediated a bifidogenic modulation of the gut microbiota, suggesting prebiotic activity. The increases in bifidobacteria were noted by the means of plate count. This study had a wide variation due to unavailability of standards for measurement of fecal samples. Polydextrose also acts as a prebiotic in a dose-dependent decrease in bacteroides, while increase in lactobacilli and bifidobacteria (Calame et al. 2018; Elli et al. 2008; Francisco 2012).

Efforts have now been directed toward understanding the underlying mechanisms of prebiotics to modulate the immune system and improve the overall health. To increase the production of SCFAs, viz., acetate, propionate, and butyrate, other protective mechanisms have been proposed. Also, it is now known that the

prebiotics resist the colonization of pathogenic bacteria in the gut epithelium by inhibiting the adherence of pathogens (Pandey et al. 2015; Saulnier et al. 2010). The health effects of prebiotics, mainly prevention of diarrhea/constipation and stimulation of mineral adsorption, are indirectly mediated by the intestinal microbiota (Pandey et al. 2015; Saulnier et al. 2010).

18.2.9 Probiotics: Supplement/Pharmaceutical/Specialist/Generalist?

The county and form in which probiotics are sold and/or are administered are usually based on the probiotic products (Baldi and Arora 2015). Further, probiotics are classified into several different entities, viz., dietary supplements, foods and its components, and pharmaceuticals. Each of these entities is subjected to different regulations and thus, the proofs regarding the demonstration of a health benefit along with safety (Baldi and Arora 2015). The approach to marketing probiotic products is mostly related to the regulatory framework. These frameworks are different in countries worldwide, thus affecting research approaches, product manufacturing, marketing, and labels on them. The importance of these regulatory frameworks is reflected in some recent papers (Baldi and Arora 2015). The path for research on drugs is fairly clear, but the path to provide evidence of a health benefit by a food or dietary supplement is not as obvious (Baldi and Arora 2015). Many probiotic products are marketed as foods or dietary supplements (Baldi and Arora 2015; Pandey et al. 2015; Sanders et al. 2013). Clear guidelines by regulatory authorities have to be made for drug-use end points.

There have been evidences of nonspecific effects of probiotics. The best example is of *Lactobacillus rhamnosus* GG that was isolated many decades ago. Since then, this strain has been targeted and tested in clinical trial for ameliorating viral infection, nonalcoholic fatty acid liver disease, and even NEC; in preterm infants, the benefits were always positive. The strain also has technological attributes such as biological robustness, adherence to epithelial cell of intestine, and inhibition of other bacteria (Horvath et al. 2011; Ritze et al. 2014; Yan and Polk 2014). Since GG was not isolated from any such target, its effects have been immense.

18.2.10 Probiotics as Bacteriotherapy

The link of microbiome alterations with a shift from health to disease seems clear, but response to probiotic-induced microbiome changes is still largely unknown (Sanders et al. 2013). Unless a clear healthy microbiome is not defined, providing a microbiological target in terms of probiotics is difficult. Beneficial effects of such interventions cannot be described in the context of physiological or clinical improvement, unless these effects are understood through the gut microbiome perspective. Few gastrointestinal clinical targets have been identified such as pediatric rotavirus diarrhea, antibiotic-associated diarrhea, *C. difficile*-associated diarrhea, ulcerative colitis, pouchitis, IBS, and NEC, where effects of probiotics have been shown to

improve the health (Ghosh 2004; Grover et al. 2012; Praharaj et al. 2015; Sanders et al. 2013; Soccol et al. 2010). Additional interventions have also been recognized beyond the gastrointestinal tract such as diabetes, metabolic syndrome, and obesity. However, it is now indeed important to study the effect of these interventions on the dynamics of gut microbiome and its stability to maintain the healthy state.

The future is now waiting for genetically modified microorganisms that can provide suitable targets for effective oral vaccine delivery and improve vaccination. Probiotic with altered cell surface components (lipoteichoic acid) could provide a potential strategy for the treatment of many inflammatory intestinal disorders (K. W. Kim et al. 2017a; Mohamadzadeh et al. 2011; Sanders et al. 2013). The use of fecal microbiota transplants (FMT) has now become a successful protective measure to manage *C difficile* infections, IBD, IBS, and with some level of success in diabetic patients (Choi and Cho 2016). However, mixtures of defined microbes imparting key functionalities will provide a more acceptable approach.

More effective probiotic interventions for microbiota-associated conditions require a deeper understanding of the interactions between genetic, microbial, and environmental influences within individuals. Such an approach will also facilitate the identification of subsets of patients most likely to respond to manipulations of the gut microbiota (responders) and the optimal agents to use in an individual subject. Probiotics have been proven promising in treatment of few diseases and also in reducing the associated risk. The need for developing probiotic as a biomarker would greatly advance this field, and measuring these physiological changes in healthy populations has to be monitored. The effects of widespread use of probiotic products have shown to be safe and effective in common infectious diseases. Today, development of probiotics to address microbiota-associated conditions has moved beyond the narrow list of microorganisms, which were previously used as probiotics.

18.2.11 Next-Generation Probiotics

Currently, the probiotic organisms have been mainly isolated from the traditional fermented foods and gut (Fijan 2014). Thus, the majority of the probiotics are from a limited list of genera, which mainly include *Lactobacillus* spp. and *Bifidobacterium* spp. (Fijan 2014). These species have been widely accepted with the status of Generally Recognized as Safe (GRAS) in the USA and Qualified Presumption of Safety status by the European Food Safety Authority (EFSA). Other probiotic species include *Bacillus* spp., *Weissella* spp., *Enterococcus, Escherichia coli*, and *Saccharomyces* present in market (Fijan 2014). Advances in the sequencing technologies have improved the current knowledge about human gut microbiome in terms of its composition and function (Langdon et al. 2016; Sweeney et al. 2015). This has led to the discovery of the extended range of organisms with potential health benefits. These potential bacteria are still at the very early stage of investigation and can be referred to as next-generation probiotics (NGPs) (O'Toole et al. 2017). The USA has termed NGPs as live biotherapeutic products (LBPs) in their new regulatory framework (Brodmann et al. 2017; Cani and de Vos 2017).

One such interesting case noted through few research studies is the classic case of *Faecalibacterium prausnitzii*. It was found to be depleted in individuals with IBD (Brodmann et al. 2017; Cani and de Vos 2017; Hemarajata and Versalovic 2013; O'Toole et al. 2017). Thus, it was interesting to find the link between disease and the absence of this organism. Feeding this bacterium to the diseased individual will promote healthy features; thus it may be considered an NGP (Brodmann et al. 2017; Cani and de Vos 2017; Hemarajata and Versalovic 2013; O'Toole et al. 2017). These observations hold true in animal models with IBD due to induction of anti-inflammatory cytokines; however, there are no evidences from clinical trials. The next best example is *Bacteroides fragilis* (strain ZY-312), isolated from the faces of infant (Brodmann et al. 2017; Cani and de Vos 2017; Hemarajata and Versalovic 2013; O'Toole et al. 2017). This bacterium possesses potential health-promoting phenotypes, viz., production of antimicrobial molecules and phagocytic substances in macrophages on incubation with colonocytes (Brodmann et al. 2017; Cani and de Vos 2017; Hemarajata and Versalovic 2013; O'Toole et al. 2017).

Numerous studies with *Akkermansia muciniphila* have been carried out since its discovery in 2004, and its abundance has been associated with beneficial effects in numerous animal models, viz., gnotobiotic and specific immune double knockout models (Brodmann et al. 2017; Cani and de Vos 2017; Hemarajata and Versalovic 2013; O'Toole et al. 2017). It has shown profound effects to improve health in metabolic disorders associated with obesity, diabetes, and liver diseases (Brodmann et al. 2017; Cani and de Vos 2017; Hemarajata and Versalovic 2013; O'Toole et al. 2017). Further studies have been conducted to find if the pasteurized bacteria and the bacterial constituents (small 30-kDa Amuc_1100) have been providing additional benefits (Brodmann et al. 2017; Cani and de Vos 2017; Hemarajata and Versalovic 2013; O'Toole et al. 2017). Such efforts in understanding the functions and the underlying mechanisms have opened a new door to putative development of drugs.

Although an exponential increase in exploring NGPs as potential probiotics has been noted, due to their novel functions, there are no studies reported through clinical trials in humans. It is indeed an important task to prove the safety and efficacy of these novel probiotics compared to the available probiotics, which belong to microbial groups (lactobacilli and bifidobacteria) well known for their safe use. However, *A. muciniphila* and *F. prausnitzii* have now shown to be safe and possess potential probiotic properties, providing interesting opportunities in the future (Brodmann et al. 2017; Cani and de Vos 2017; Hemarajata and Versalovic 2013; O'Toole et al. 2017).

18.2.12 Need of the Hour: Population-Specific Probiotics

The gut microbiome is influenced by several factors, and recent reports have highlighted the population-specific microbiome in geographically separated populations (Gupta et al. 2017; Shin et al. 2016; Voreades et al. 2014). Considering a case, consuming soya-rich diets confers positive health benefits for many diseases mainly cancer and cardiovascular disease in humans. S-equol is produced from the

soya isoflavone daidzein by bacterial enzymes and not from human enzymes (Liu Po Hung 2014; Tham et al. 2011). In a study carried out in humans with diet rich in soya foods, only 30% of the Western population could produce S-equol as compared to the 60% from Japan, Korea, or China (Liu Po Hung 2014; Tham et al. 2011). Thus, the benefits described in Asian population cannot be generalized to the Western population due to the difference in the gut composition. Similarly, the metabolism of widely used analgesic acetaminophen to acetaminophen sulfate or glucuronide is dependent on the gut composition (Ben-Shachar et al. 2012). These studies thus indicate that the results of any drug trial carried out in one population may not be generalized to a population having different gut composition (Ben-Shachar et al. 2012; Liu Po Hung 2014). Such studies have implications in the development of vaccines and antibiotics (Ferreira et al. 2010; Nakaya and Bruna-Romero 2015; Praharaj et al. 2015), probiotic products for specific human populations (Arboleya et al. 2012; Grover et al. 2012), and information designing the medical interventions (De Filippis et al. 2018; Lam et al. 2018).

Recent microbiome studies have clearly highlighted the differences in microbiome between the populations across the globe. Thus, population groups with particular microbiome present particular nutritional needs and thus constitute an opportunity for the development of specific probiotics that fulfill the requirements of such groups. The import of probiotics in Indian market is observed to be higher; however, considering the abovementioned facts (Soccol et al. 2010), population-specific probiotic formulations are the need of the hour.

Few research studies have now been directed toward developing such population-specific probiotics; *Enterococcus faecium* strain 17OM39 is one of such kind. An integrated approach of in vitro experiments, genomic analysis, and comparative genomics to characterize the metabolic characters of strain 17OM39 revealed features that are important for the organism's intestinal residence and its inherent potential of being probiotic (Vikas C Ghattargi et al. 2018a, b). This bacterium showed a strong carbohydrate metabolism and essential amino acid and vitamin production. Although, no in vivo studies have been published for this strain, it is indeed important to explore its functions in detail to understand health benefits imparted by such novel and indigenous probiotics.

18.3 Microbial Interventions: Fecal Microbiota Transplantation (FMT)

Human intestine, lined by microbiota, offers protection against pathogens and provides gut homeostasis. Any imbalance in the composition or relative proportion of gut microbes may affect host metabolism, which may lead to several gastrointestinal diseases (GI) as well as non-gastrointestinal disorders (Hooper et al. 2012). Factors like dietary changes, over medication, and infections could alter gut homeostasis by disrupting the microbial lining. Of these, antibiotic-driven dysbiosis (imbalance) has a major role in the disruption of normal gut flora by reducing their colonization resistance. Such disruptions lead to the development of gastrointestinal

disorders (GI) such as *Clostridium difficile* infection (CDI), inflammatory bowel's disease (IBD), enterocolitis, irritable bowel's syndrome (IBS), diarrhea, and idiopathic constipation and metabolic disorders like insulin resistance and obesity. Moreover, dysbiosis can also trigger certain autoimmune disorders such as celiac disease, autism etc. (de Groot et al. 2017). Bacterio-therapeutics could revert this by reinstalling the proportion of healthy microflora in patient's gut either by microbial transplantation or by pre−/probiotic administration (Smits et al. 2013).

18.3.1 The Concept

Fecal microbiota transplantation (FMT) is a technique to acquire beneficial microbiota by the direct administration of the fecal suspension collected from a healthy donor in order to restore the gut microbiota in patients with severe GI disorders (Johan and Bakken 2011; Smits et al. 2013). "Simple rationale behind FMT is that the intake of fecal flora from a healthy donor helps in the re-establishment of healthy bowel function by restoring normal gut flora in recipients" (Johan and Bakken 2011). Apart from FMT, other microbiota transplantations like vaginal, skin, oral, and nasal could work, but are not that advent in dealing with the diseases as FMT does. Here, we describe the evolution of the technique and few diseases where FMT has proven to be a promising approach.

18.3.2 History of Fecal Microbiota Transplantation

Ancient medical practices by Ge Hong, in China around the fourth century, reported the first use of fecal therapy to treat diarrhea patients, and it is named as "yellow soup." As per the Chinese handbook of emergency medicine "Handy Therapy for Emergencies," the fecal therapy worked like a miracle that brought patients back from the edge of death (Zhang et al. 2012). It was unexplored for several centuries; nevertheless, a published report in the sixteenth century reveals the application of infant feces and fermented fecal infusions (as fresh and freeze-dried forms) to cure severe diarrhea, fever, vomiting, and constipation problems by Li Shizhen (Sbahi and Di Palma 2016). Later in the seventeenth century, an Italian anatomist adopted this technique in veterinary medicine, named as "transfaunation" (F. Zhang et al. 2012). In Africa during World War II, German soldiers had used warm camel stool for the treatment of bacterial dysentery (Uwlfoh et al. 2016). Successful execution of FMT via fecal enema in modern medicine started from 1958 to cure pseudomembranous enterocolitis patients with antibiotic-associated severe diarrhea, which was later understood as *C. difficile* infection (CDI) (Choi and Cho 2016; Zhang et al. 2012). Over the past three decades, FMT has been widely explored in dealing deadly recurrent diseases (Fig. 18.1) as an alternative approach to conventional antibiotic practices in a cost-effective manner.

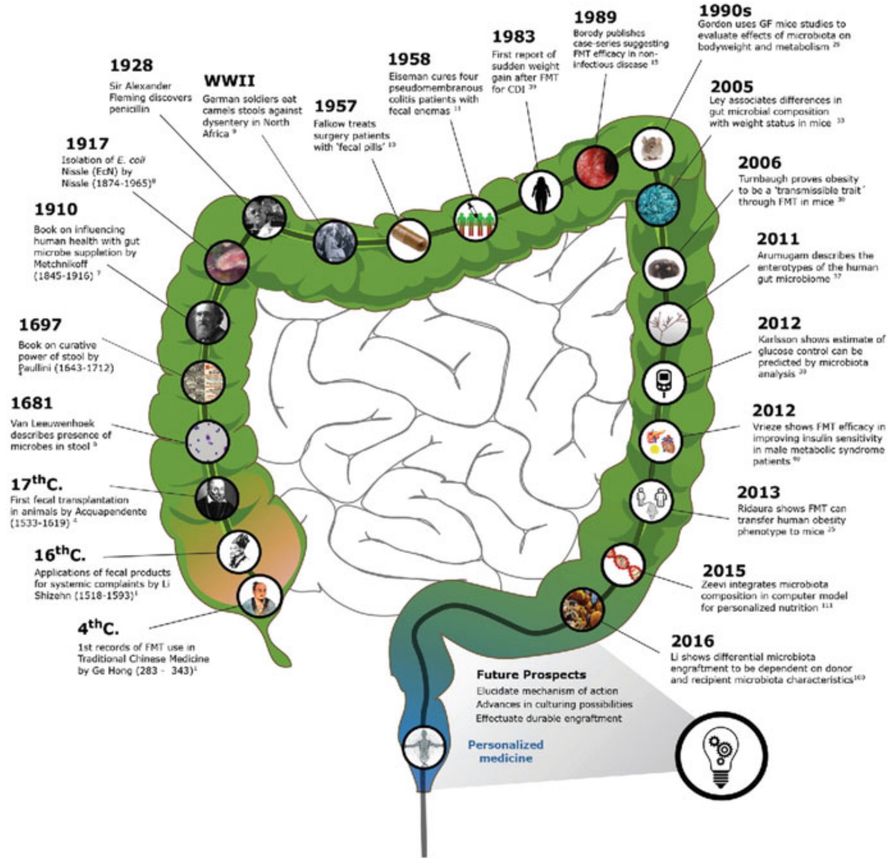

Fig. 18.1 Era of evolution and history of fecal microbial transplantation (de Groot et al. 2017)

18.3.3 Fecal Microbiota Transplantation in Diseases

Reports provide knowledge on FMT as an emerging approach in the management of certain GI disorders and few non-GI disorders like metabolic, autoimmune, allergic, and neuropsychiatric disorders.

18.3.3.1 *Clostridium difficile* Infection

C. difficile infection (CDI) is a severe nosocomial infection that contributes to 25% of overall antibiotic-associated diarrhea cases (Brandt et al. 2012). Symptoms range from mild diarrhea to potentially lethal pseudomembranous colitis. Conventional treatment includes a cost-associated antibiotic course with the oral administration of metronidazole or vancomycin (or fidaxomicin) (Weiss et al. 2011). At this stage, most of the cases could recover; rather, only few cases (15–30%) developed relapsing or recurrent CDI (rCDI), as the antibiotic usage led to the dysbiosis of normal gut flora and allowed the enriched growth of pathogenic *C. difficile* (Bakken

2009)(Aroniadis and Brandt 2013). Additional courses of oral vancomycin or vancomycin in a pulsed and/or tapered regimen are used to treat rCDI. Rate of recurrence on the second exposure to antibiotics was up to 65% (Brandt et al. 2012). The reason for this high rate of recurrence might be the spore formation in *Clostridium* or the horizontal transfer of vancomycin-resistant genes from intestinal *Enterococci* to *Clostridium* spp. The colonoscopic FMT is recommended to such cases as it could be the effective first-line therapy for both CDI and rCDI with an overall primary cure rate of 91% and a secondary cure rate of 98% (Bakken 2009; Johan and Bakken 2011).

18.3.3.2 Inflammatory Bowel Disease (IBD)

IBD is a global, idiopathic inflammatory disorder of gastrointestinal tract. IBD outcomes are mainly of two forms, i.e., Crohn's disease (CD) and ulcerative colitis (UC). Poor knowledge and rapid prevalence in industrially developed countries are now gaining global attention. According to the Centers for Disease Control and Prevention (CDC-USA) reports, about 1–1.3 million people in the USA are suffering with IBD (Epidemiology 2004; Kappelman et al. 2007).

Crohn's disease (CD): It includes the inflammation along the lining of digestive tract from mouth to anus; in severe cases, it may spread to the deeper tissues that are associated with the tract.

Ulcerative colitis (UC): It is a chronic inflammatory disease of large intestine, which involves development of ulcers and persistent inflammation in the innermost layer of the colon and rectum. Since it shares clinical manifestations with Crohn's disease, these two often get confused to deem the therapeutic recommendations even if they are distinct in their pathophysiological effects (Bachmayer 2012).

Severe complications may lead to colon cancer, rigorous dehydration, and inflammation in bile ducts and will cause gradual liver damage. The contribution of gut microbial dysbiosis to IBD is still unclear as it is the cause or consequence of the disease. However, provided evidences with the depletion of *Bacteroides* and *Lachnospiraceae* group (within the *Firmicutes* phylum) and a relative enrichment of pro-inflammatory bacteria such as Proteobacteria and Actinobacteria suggest the role of dysbiosis in the development of IBD (Aroniadis and Brandt 2013). In addition, inflammatory mediators like interleukins (IL) and interferons (IFN) generated by the unconditional immune responses during the infection will disrupt the microbial colonization of intestine, resulting in the chronic inflammation and development of mucosal lesions (Patel et al. 2018). Several studies reported FMT as a promising approach for the management of diseases associated with gut dysbiosis, including CD, UC, and IBD. In 1989, FMT by retention enema was first reported for IBD, and it could cure the disease successfully in 6 months (J. D. Bennet and Brinkman 1989). A systematic review and meta-analysis analyzed 18 studies (9 cohort studies, 8 case studies, and 1 RCT) on FMT that included 122 patients with IBD (79 UC, 39 CD, and 4 unclassified). In this study, few case series are excluded to minimize publication bias, and the overall clinical remission was about 22% in UC, 60.5% in CD, and 64.1% in young patients (age 7–20 years), respectively(Choi and Cho 2016).

18.3.3.3 Irritable Bowel Syndrome (IBS)

IBS is a globally prevalent (10–20%) functional GI disorder (FGID) with multifactorial etiology, with recurring symptoms of abdominal pain, low-grade inflammation, and visceral hypersensitivity along with alteration in gut motility and gut-brain axis (Forbes et al. 2011). IBS is subclassified based on the predominant stool (ROME III criteria) that is used: diarrhea predominant (IBS-D), constipation predominant (IBS-C), mixed (IBS-M), and un-subtype (IBS-U) (Mearin and Spiller 2006). Reports of IBS in patients with increased risks of CDI, acute gastroenteritis, idiopathic constipation, and *Shigella* infections make the role of gut microbial dysbiosis in the development of IBS evident (Aroniadis and Brandt 2013; Bennet et al. 2015; Johnsen et al. 2018). It is discriminated with increased proportion of *Firmicutes* to *Bacteroidetes*, *Ruminococcus* spp., *Dorea* spp., and *Clostridium* cluster XIVa phylotypes and reduction of Actinobacteria forms including bifidobacteria (frequently used as a probiotic) from normal gut microbiota (Ahmad and Akbar 2016). A study reported nearly 90% of the recovery rate with an improvement in defecation and decreased abdominal bloating, among which 60% showed long-term benefits for about 9–19 months post FMT (Kelly et al. 2015). Besides these encouraging results, it is important to conduct randomized controlled trails (RCT) for large cohorts in long-term concerns.

Low-grade inflammation developed by the deposition of lipopolysaccharides (LPS) of enriched gram-negative bacteria may lead to the induction of other metabolic consequences. Few metagenomic studies describe the synchronization of specific gut microbial changes with the metabolic disorders, including diabetes mellitus, obesity, hypertension, chronic fatigue syndrome (CFS), etc.

18.3.3.4 Glucose Homeostasis

WHO declared that diabetes is one of the most prevalent global diseases rapidly growing in developed countries like the USA, the UK, etc. Microbial interference for instance, "insulin sensitivity" boosted by *Akkermansia municiphila*, *Faecalibacterium prausnitzii* and "insulin resistance" developed by the domination of *Prevotella copri*, *Bacteroides vulgatus* which in turn develops hypoglycemic and hyperglycemic states respectively (de Groot et al. 2017). Simple enrichment of *Akkermansia* population by FMT could be an alternative to the harsh medication of diabetic patients. Of note an example of a commercial drug "metformin" improving glucose homeostasis by increasing the population of *Akkermansia* species in diet-induced obese mice models (Everard et al. 2013; Smits et al. 2013).

18.3.3.5 Obesity

It is a multifactorial, metabolic syndrome, rapidly increasing in prevalence approximately three times in last the four decades. Exact mechanism for the development of obesity is unknown, but diet, physiological, and genetic factors are the only believed conceptual factors. However, animal experiments (Ridaura et al. 2014) provided insight into the role of gut microbiota and the influence of disordered gut homeostasis on the overall metabolic function that ultimately leads to obesity. *Bacteroides uniformis*, *Parabacteroides merdae*, and *Alistipes putredinis* are the

obesity-associated gut microbiota which help in suppressing adiposity by elevating the levels of acetate, propionate, and butyrate (Ridaura et al. 2014). Unfortunately, it is believed that obesity could be one of the post-FMT consequences (Campbell et al. 2015).

18.3.3.6 Chronic Fatigue Syndrome (CFS)

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a serious, long-term illness that affects many body systems. Symptoms include severe fatigue and sleep problems, pain, and dizziness, and it may lead to gradual paralysis (post-exertional malaise [PEM]) (Aroniadis and Brandt 2013). The Institute of Medicine (IOM) reported in 2015, about 0.83–2.5 million US populations estimated to have ME/CFS, but remain undiagnosed. The level of *Escherichia coli* significantly decreased in the CFS group when compared to the healthy controls (92.3% vs. 49%), providing a scope for bacteriotherapy (Aroniadis and Brandt 2013; Evrensel and Ceylan 2016). Response rate to colonoscopic FMT with 13 strains was 70%. Long-term observations revealed 58% full recovery until 15–20 years and 41% with no recurrence for 1.5–3 years (Evrensel and Ceylan 2016).

FMT could deal with other metabolic syndromes related to gut microbial interactions like atherosclerosis, hepatic steatosis, and hypertension (de Groot et al. 2017). Furthermore, gut microbiota is associated with many non-GI disorders, including autoimmune and neuropsychiatric disorders, where FMT could be the confronting approach.

18.3.3.7 Autoimmune Disorders

There are proven reports on the shift of intestinal microbiota inducing autoimmune disorders like arthritis, idiopathic thrombocytopenic purpura (ITP), celiac disease, autism, Sjogren’s syndrome, and Hashimoto’s thyroiditis. For example, Hashimoto’s thyroiditis can be triggered by *Yersinia enterocolitica*. Xu (2015) has reported post-FMT recovery in ulcerative colitis case with ITP, with the normalized levels of platelets (Evrensel and Ceylan 2016).

18.3.3.8 Neuropsychiatric Disorders

Strong evidences on the role of gut dysbiosis in the etiopathogenesis of certain neuropsychiatric disorders like autism, Parkinson’s disease, and multiple sclerosis support the efficacy of FMT as an intervention (Evrensel and Ceylan 2016). There are no such clinical studies on autistic patients. However, they are deviated by the presence of rich and diverse types of *Clostridium* species from a normal gut profile. In a study by Borody, practical recovery is seen in autistic children by daily FMT of cultured *Bacteroidetes* and *Clostridia* for several weeks (Aroniadis and Brandt 2013). It has been proven in mice models treated with Bacteroides fragilis by the elevated expression of tight junction proteins.

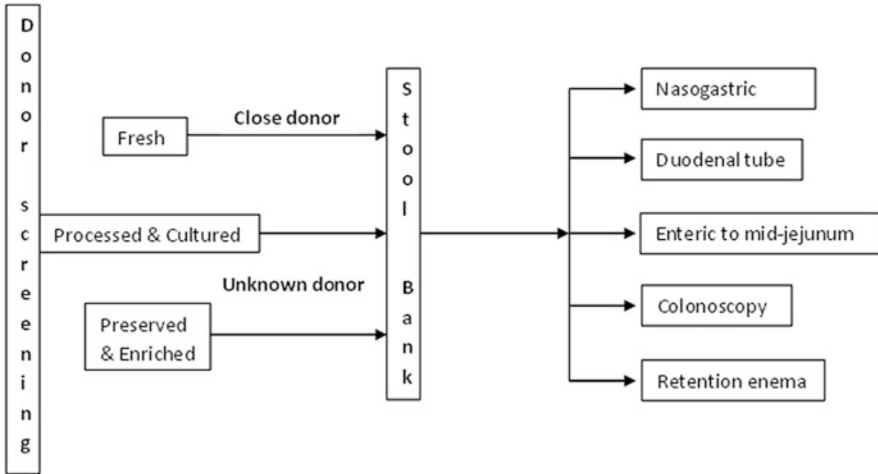

Fig. 18.2 Overview of fecal microbial transplantation (FMT) procedure

18.3.4 Regulations

Regulations vary around the world for FMT. For example, there are no regulations in the UK, whereas in the USA, the Food and Drug Administration (FDA) and European Consensus Conference on FMT have approved the usage, but confined to CDI and rCDI (Cammarota et al. 2017). Evidence-based recommendations that strengthen FMT in clinical practices are a European guideline "FMT is strongly recommended (A-I) after a second recurrence of CDI" and a more cautious recommendation from the American College of Gastroenterology, according to which "on the third recurrence after a pulsed vancomycin regimen, FMT should be a conditional recommendation" (Kappelman et al. 2007). There are no such globally approved regulations for ideal FMT procedure, but the National Institute for Allergy and Infectious Diseases (NIAID) committee along with FDA had discussed few criteria for donor selection, sample screening, and preparation as mentioned in Fig. 18.2 (Epidemiology 2004). These are to avoid possible risks and improve the quality, efficacy, and safety.

18.3.5 Procedure

18.3.5.1 Donor Screening

Composition of gut microbiota is individual-specific, and within individuals, it varies in different body parts; hence, a donor can be either from a closed group (maternal relation or distant relation with same kind of lifestyle) or utterly unknown. New approaches are under trial to generate a universal "super donor," by the composite sample from mixed donors. The criteria to select potential donor are still under debate. However, the quality of a potential donor cannot be restricted to

richness in the diversity of gut microbiota or the marker microbes that are absent in patients. There is a need to generate a globally accepted donor exclusion criteria for safe practices (Hoffmann et al. 2017). The majority of regulations concerns avoiding the risk of transmissible infections (viral, bacterial, and parasitic) or other metabolic disorders during this process. Few case studies of FMT from a daughter to mother to cure diarrhea helped in the development of obesity besides the recovery of the patient (Campbell et al. 2015), similar effect was found in mice model as well (Ridaura et al. 2014), and a fecal transplant from a lean donor induced insulin sensitivity in recipient (Vrieze et al. 2012). Despite these, few FMT patients developed depression and bowel disruptions, with no proper evidences they are doubtful yet. Therefore, an ideal donor should be healthy with no prior medical history of having autoimmune diseases, metabolic diseases, malignancy, and any communicable diseases in their family. To achieve this, a proper survey regarding age, sex, health status, and others along with the frequent health screening is required prior to the procedure, such as stool test (to trace donor's gut profile and history of GI disorders) and blood test (to check history of viral or bacterial infections and allergic reactions) (Table 18.1) (Aroniadis and Brandt 2013).

18.3.5.2 Sample Preparation

Influence of sample on the efficacy of FMT is strongly supported by the following reports (Fig. 18.3) (Choi and Cho 2016). Reports of few randomized controlled trials for the treatment of UC by FMT by Moayyedi and Rossen showed that prolonged exposure of stool sample to aerobic conditions might affect the viability of certain group of bacteria in the inoculums, allowing the other group to flourish and colonize recipient's intestine (Moayyedi et al. 2015b). For example, a study by Kump reported a significant increase of fusobacteria in post-FMT patients (Cui et al. 2016).

Manipulation of stool samples from basic frozen form (e.g., frozen stool, freeze-dried stool) to sophisticated products (such as RePOOPulated synthetic stool and capsules) is necessary to develop a better way to commercialize, transport, and easily consume rather than a whole fecal administration. This is a targeted approach that enables greater specificity and quality control by purifying specific bacterial strains (consortia) from stool samples. For example, SER-109 is to treat rCDI, which is under clinical trials by Seres Therapeutics. Stool banks such as OpenBiome and Advancing Bio help in screening of frozen material ready for clinical use. There are filtered and cryopreserved products, for example, RBX2660 developed by Rebiotix and a lyophilized powder by CIPAC Therapeutics as rectal infusion (Hoffmann 2017; Kelly et al. 2015). Ecobiotic products comprise purified and enriched bacterial spores from stool and are packed as capsules; they are used to suppress the gut dysbiosis. Several pharmaceutical companies and biotechnology start-ups are developing various stool-based products to treat rCDI and other GI disorders to improve quality and specificity. Vedanta Biosciences are identifying and culturing the decreased/missing bacterial strains or the strains that have been shown to suppress chronic gut inflammation during CDI and rCDI, as a part of drug development (Hoffmann et al. 2017; Kelly et al. 2015). The Norwegian study revealed that the transplant formulation has laxative effect arising from the glycerol content.

Table 18.1 The FMT donors are required to follow the mentioned questionnaire and tests prior to the sample collection for a potential screening (Choi and Cho 2016)

Survey (donor + recipient) includes	Donor tests includes	
1. Information regarding the lifestyle and other general questionnaire?	Stool	Serological
2. Any exposure to antibiotics/ immunosuppressants/chemotherapeutic agents in the past 3 months?	CD toxin	Hepatitis A IgM
	Stool culture	Hepatitis B surface antigen
	Ova and parasites	Antibodies to hepatitis B surface antigen
3. Any clinical history of chronic diarrhea, constipation, IBD, IBS, colorectal cancer/ ulcers, obesity, immunocompromised diseases, any metabolic or chronic fatigue syndromes, and any communicable diseases in their family	For enteric pathogens	Hepatitis C antibody
	Giardia antigen	HIV type 1 and 2 antibody
	H. pylori antigen (for oral administration only)	Syphilis
	Cryptosporidium antigen	General parameters
	Isospora (acid fast stain)	
	Rotavirus	
4. Had tattoos or any body piercings in past 3 months?	Fecal occult blood testing	
5. Information regarding recipient clinical history, especially about allergies? (if so, donor has to avoid ingesting them for at least several days before the transplantation.)		

*Patel et al 2013, reported a successful outcome in two patients with recurrent CDI unresponsive to repeated courses of antibiotics who received a **stool substitute**, a preparation of **33 different intestinal bacteria isolated in pure culture from a single donor.** Recent studies demonstrated that FMT using**frozen inoculums fromscreened** volunteer **healthydonor** is effective for treating recurrent CDI. A more recent feasibility study used frozen fecal capsules, prepared from prescreened healthy donor for treating 20 patients with recurrent CDI. The results showed an overall 90% rate of clinical resolution of diarrhea after one or two treatment courses.The **outcomes** of this approach was **similar** with those of FMT **with fresh stools, suggesting** that **preparation of frozen transplants can simplify the practical aspects of FMT without loss of efficacy or safety**. In Korea, Gweon et al, first reported two cases of **refractory pseudomembranous colitis** treated with FMT in 2013. Subsequent case reports showed **FMT can cure CDI complicated by acute respiratory distress syndrome, toxic megacolon by** CDI, and CDI **in a patient colonized by vancomycin-resistant enterococcus.***

Fig. 18.3 Reports supporting the influence of sample preparation in overall efficacy of FMT as a concern for the wide range of applications (Choi and Cho 2016)

Manipulation strategies of stool arguably could have implications for their regulation. Current regulations are proposed to reduce the glycerol content, and the ideal transplant retention time is between 30 minutes and 2 hours for a better retainment in the recipients (Hoffmann et al. 2017).

18.3.5.3 Bowel Preparation

A clear medical history of recipients could help to avoid unwanted allergic reaction to fecal infusion (Choi and Cho 2016). Recipients should take antibiotic course until 2–3 days prior to FMT. A bowel preparation (evacuating food from respective part of GI tract) on the day before the procedure is necessary for all patients, regardless of route. Fresh stool is most often used within 8 hours of passage; however, frozen stool samples have to be thawed and administered within 1–8 weeks after passage (Aroniadis and Brandt 2013; Choi and Cho 2016).

18.3.5.4 Stool Preparation

Stool is a complex biological material commonly suspended in sterile saline; however, water and other diluents like yogurt and milk are also used without consistent differences in resolution or relapse rates. It is believed that there is a positive correlation of improved outcome with larger volumes (Aroniadis and Brandt 2013; Brandt et al. 2012). The ideal fecal amount to be used for FMT has not been standardized; however, less than 50 g of infusion is used to get fourfold greater relapse rate (Aroniadis and Brandt 2013; Choi and Cho 2016). Prior to the aspiration into syringe, the suspension has to be sieved through gauze pads to remove large particulate matter (Aroniadis and Brandt 2013).

18.3.5.5 Delivery System

Various factors such as route of administration, procedure, and duration of treatment could influence the efficacy and effectiveness of FMT. Until 1989, fecal microbiota was commonly administered via retention enema. Subsequent developments of other fecal infusion techniques are as follows: gastroscope or nasogastric tube (1991), duodenal tube, enteric tube to mid-jejunum, colonoscopy (2000), and self-administered enemas (2010) (Choi and Cho 2016; Johan and Bakken 2011). However, repeated infusions of fecal enema and single infusions of colonoscopy are most common and effective among others (Aroniadis and Brandt 2013; Brandt et al. 2012). Since colonoscopic FMT is safe, easy, rapid, and well tolerated, allowing the inspection of the entire colon and ileum (unlike enemas which only reach the splenic flexure) gives better elucidation of disease extent and severity (Brandt et al. 2012). For example, colonoscopic FMT contributes about 80% cure rate of rCDI, whereas through nasogastric tube 60% cure rate is achieved. However, it is necessary to take extra care for the patients suffering from colon-related infections to avoid unwanted perforations (Choi and Cho 2016).

18.3.6 Adversities

FMT is considered as a therapy but not a treatment! It restores the phylogenetic richness of the recipient's gut microbiota without prolonging the perturbation of the normal flora. Despite the high success rate in treating rCDI (>90%), FMT is still in its infancy in medical exercises because of the risks of pathogenic transmission, limited viability of fresh samples, diverse strategies of donor screening with high risk of bias, stool processing, discomfort in the overall procedure, and lack of awareness (Smits et al. 2013). Theoretically, FMT is used to restore certain dysbiosis, in which one could expect opposite situation as an adverse outcome. For instance, FMT from a donor with obese phenotype could potentially transmit obesity to the recipient (Campbell et al. 2015). A long-term follow-up of colonoscopic FMT in rCDI patients by Brandt for ≥ 3 months revealed that 4 out of the 77 patients reported a new medical condition after FMT including peripheral neuropathy, Sjogren's disease, idiopathic thrombocytopenic purpura, and rheumatoid arthritis (Brandt et al. 2012). Hence, well-designed, long-term, and large randomized controlled studies are needed to determine the safety and efficacy of FMT in the management of GI disorders (Kelly et al. 2015). Long-term exposure of sample to aerobic conditions would affect the viability of the sample, allowing the unwanted abundance and colonization of certain bacteria after FMT, which may lead to adverse outcomes (Cui et al. 2016).

Since stool is a complex biological material, targeted manipulations (purification and enrichment) in fecal infusions could achieve better outcomes. This could lead to more precise approach by making synthetic stool with selected and screened consortium (de Groot et al. 2017; Hoffmann et al. 2017). Knowledge-based studies on these specific consortium and related diseases could pave a new way to the application of beneficial bacteria/consortium (pre- or probiotics) as personalized therapeutics (Patel et al. 2018).

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Microbiome: A Source of Novel Bioactive Compounds and Antimicrobial Peptides

19

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Abstract

The human body is an abode for trillions of microbes that have co-evolved with humans and are beneficial in several ways. Human oral cavity, gut, and skin have been shown to harbor abundant and diverse microbial communities. Other body parts, some of which were previously considered to be sterile, have also been shown to possess a number of resident bacteria. The microbial inhabitants on or in human body are largely commensals. Many of these organisms produce small bioactive molecules or peptides that interact with other bacteria to inhibit/modify their growth and colonization or with the host to modulate the host immune response. This chapter briefly discusses the microbial diversity of human body and presents a detailed account of antimicrobial peptides and bioactive molecules identified from these microorganisms and their potential applications.

Keywords

Microbiome · Bioactive compounds · Bacteriocin · Antimicrobial · Immunomodulatory · Anticancer · Human metagenome · Biosynthetic cluster

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19.1 Introduction

The human body houses a rich diversity of microbes. A variety of microorganisms including bacteria, archaea, viruses, fungi, and mites reside in different areas of the human body forming the human microbiome. Approximately 3.8×10^{13} microbes are present on/in a healthy adult human being at a time. These microbial cells slightly outnumber human cells (1.3:1) (Sender et al. 2016). The microbes may live in a symbiotic association or as commensals with the host. Microbial composition varies among different individuals, at different sites, and from time to time (Parfrey and Knight 2012). The body sites exposed to the environment including the skin and nasal canal are more variable than internal sites. Mouth is considered to be the most diverse niche followed by gut. There are high levels of variability among individuals, but an individual's microbiome has a relative stability at different times (Costello et al. 2009). On the other hand, a few body sites previously considered sterile have now been shown to harbor microbes even in the healthy state including the lungs, bloodstream, and placenta (Lloyd-Price et al. 2016). Microbial colonization at a body site may be a function of the immunity, gender, age, genetic, and ethnic predisposition of the host and environmental factors (Kong 2011; Ley et al. 2006).

19.2 Human Microbiome and Its Importance

In the early 1900s, Elie Metchnikoff hypothesized that the intestinal microbes play a role in maintaining health and longevity. He promoted the use of probiotics in the form of fermented food (Metchnikoff 1908). This theory was disputed at that time but resurfaced in the early 1990s and gained importance (Mackowiak 2013). Currently, probiotics or products produced by probiotic bacteria are employed as supplements for many disorders owing to their importance in maintenance of the healthy microbiome. The healthy human microbiome varies between different organs like the gut and the skin and also at different locations on the same organ such as dry and moist sites on the skin (Lloyd-Price et al. 2016). Antibiotic exposure along with lifestyle changes alters the microbiome (Dethlefsen and Relman 2011).

The human gut is a diverse niche containing approximately 400 species in an individual (Lloyd-Price et al. 2016). Diet, host factors, and nutritional status of an individual shape their gut microbiota. The microbes have a heterogeneous distribution across the gastrointestinal tract that is developed during the early years. Bacteriodetes and Firmicutes dominate the gut (Turnbaugh et al. 2006). In the distal esophagus, *Streptococcus*, *Veillonella*, and *Prevotella* spp. dominate (Pei et al. 2004). The stomach has species belonging to the phyla Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Fusobacteria (Bik et al. 2006). Apart from Bacteriodetes and Firmicutes, the colon also harbors Actinobacteria, some genera belonging to Verrucomicrobia and Proteobacteria (Lloyd-Price et al. 2012). Anaerobic Archaea including *Methanobrevibacter smithii*, a few viral and

fungal species are the other microbes present. The structure of the community is fairly resilient to changes including antibiotic administration unless exposed to long-term dietary changes (Sekirov et al. 2010).

The oral niche is home to more than 700 bacterial species and other microorganisms (Aas et al. 2005). These microbes are present in the form of a biofilm whose composition changes with time. The initial colonizers include *Streptococcus mitis* and *Streptococcus mutans*, which further prepare the surface for late colonizers including anaerobic gram-negative cocci and oral archaea (Wu et al. 2010).

Nose or the anterior nares remain constantly in contact with the external environment thus a home to transient microorganisms. The microbial composition is shaped by interaction with host factors including nutrient limitation, other microbes, and the environment. Gram-positive cocci including *Peptostreptococcus*, *Staphylococcus*, *Corynebacterium*, and *Streptococcus* are common residents. Other microbes including *Propionibacterium* sp., members of Lactobacillales, and Incertae sedis IV group were also recorded in some individuals (Wos-Oxley et al. 2010).

Skin is the outermost tissue of the human body that acts as the first line of defense against invading microbes. Human skin is colonized by a diverse milieu of symbiotic microorganisms that interact with the host immune system and may add to the defense against pathogens (Grice and Segre 2011). About a million bacteria reside on a cm^2 of the skin surface. The skin microenvironment varies in terms of pH, temperature, moisture, and sebum content, with a total of 205 genera detected and 40 species/individuals (Belkaid and Segre 2014). Microbes belonging to Actinobacteria are the most prevalent on the skin sites along with Firmicutes, Proteobacteria, and Bacteroidetes forming the majority (~98%). Members of the bacterial genera *Corynebacterium* dominate the moist sites, whereas *Staphylococcus* and *Propionibacterium* spp. are higher in the sebaceous sites on the skin. The dry sites are the most diverse and harbored a mixture of bacteria with high β-Proteobacteria and Flavobacteriales (Grice et al. 2009).

The human vagina harbors low microbial diversity with Lactobacilli forming the dominant microbiota. A total of 282 different microbial phylotypes were reported to reside in the vagina of 400 women of four different ethnicities. *L. iners*, *L. crispatus*, *L. gasseri*, and *L. jensenii* majorly dominate reproductive-aged women (as determined in a study of white, black, Hispanic, and Asian women). *Atopobium* sp., *Clostridium* sp., Bifidobacteriales, and Mycoplasma are also present in some of the women categorizing the microbiota into two groups: Lactobacilli and non-Lactobacilli. Variance of this composition in women of different ethnicities makes them prone/resilient to certain vaginal pathologies (Martin 2012; Ravel et al. 2011).

19.3 Microbiome and Antimicrobial Compounds

Microbes produce bioactive compounds in response to microbial competition and other environmental stresses. These natural products have been employed as therapeutics against human pathogens as well. These natural products had a golden era during the 1940s–1960s post discovery of penicillin. Initially obtained by screening

of soil and marine and other diverse habitats, the focus of natural product research shifted toward capturing these products from the unculturable microbial majority (Lam and Crawford 2018; Moore and Gerwick 2012). Currently, new bioactive compounds including novel antibiotics are being discovered using a combination of genome mining, activation of silent biosynthetic pathways, and metagenomic analysis of a variety of ecosystems (Challinor and Bode 2015; Garcia-Gutierrez et al. 2018; Rutledge and Challis 2015). Emerging resistance to known antibiotics or their derivatives necessitates the discovery of alternative natural products to fight bacterial pathogens. The human microbiome represents a vast resource of untapped microbes capable of producing secondary metabolites and peptides. The discovery of small biomolecules and peptides from the human microbiome may mark the next era of antimicrobial drug discovery.

Small molecules are secreted by human commensals to drive microbe–microbe and microbe–host interactions. To understand these interactions and identify potential antimicrobial compounds, small molecules were screened on a large scale from genome sequences of isolates from various body sites. Metagenomes and metatranscriptomes collected for the human microbiome project were surveyed using bioinformatics to understand the presence of these molecules in healthy humans. About 3118 biosynthetic gene clusters (BGCs) coding for small molecules were identified in 752 samples obtained from five body sites of healthy individuals. Three classes of secondary metabolites, lantibiotics, thiopeptides, and thiozole/oxazole-modified microcins were found to occur in high amounts at all sites. A few nonribosomal peptide sequence (NRPS) clusters were found exclusively in gut samples/isolates. Mostly, these BGCs were distinct from those present in microbes associated with nonhuman hosts. Interestingly, one of the BGCs detected in the oral microbiome was strikingly similar to a cluster encoding a macrolide obtained from a marine isolate. Also, eight BGCs were absent in the isolate genomes indicating an unculturable source. One of these BGCs from the metagenome encoded for a novel thiopeptide could be a potential drug (Donia et al. 2014). Since hundreds of BGCs with unknown function exist in the human microbiome, identifying and utilizing them to our advantage can help in developing novel therapeutics against a variety of infections. Table 19.1 summarizes various antimicrobial compounds produced by human associated microbes.

19.3.1 Nasal Microbiome

Nasal commensals survive in a low nutrient environment and produce an unexpectedly high diversity of bacteriocins to limit the growth of competing microbes. Out of the 89 bacterial strains isolated from the human nose, 77 could inhibit one or more of the 11 tested pathogens through the production of antimicrobial products. The *S. epidermidis* strain IVK45 produced a representative bacteriocin, i.e., nukacin, that showed strong antimicrobial activity against various pathogens. It is encoded on an easily exchangeable mosaic plasmid comprising transposases, recombinases, and DNA from various sources, which may contribute to creation of new bacteriocin

Table 19.1 Bacteriocins and antimicrobial peptides identified from human microbiome

Host site and producer	Compound	Antagonistic activity against	References
Nasal microbiome			
Staphylococcus lugdunensis	Lugdunin	Gram-positive strains including methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococci*	Zipperer et al. (2016)
Staphylococcus epidermidis IVK45	Nukacin	Gram-positive strains	Janek et al. (2016)
Oral microbiome			
Streptococcus pyogenes FF22	SA-FF22	Indicator strains I_1, I_5–I_8	Peninsula (1991)
Streptococcus salivarius 20P3	Sal A	Indicator strains I_1–I_9	Ross et al. (1993)
S. pyogenes SF370	Sal A1	Indicator strains I_1–I_9 excluding I_3, I_5, I_8	Wescombe et al. (2006)
S. salivarius JH	Sal A3-A5	Indicator strains I_1–I_9	Wescombe et al. (2006)
S. salivarius K12	Sal A2 and B	Indicator strains I_1–I_9	Hyink et al. (2007)
S. salivarius 9	Sal 9	Indicator strains I_1–I_9 except I_8	Wescombe et al. (2011)
S. salivarius G32	Sal G32	Indicator strains I_1, I_5–I_8	Wescombe et al. (2012)
Streptococcus mutans	Mutanobactin	*C. albicans*	Joyner et al. (2010)
Gut microbiome			
Lactobacillus gasseri	Gassericin	Gram-positive foodborne pathogen	Kawai et al. (1994)
Lactobacillus reuteri	Reutricin	*B. cereus*	Kabuki et al. (1997)
Clostridium beijerinckii	Circularin	*Clostridium* spp. mainly *C. tyrobutyricum*	Kawai et al. (2004)
Lactobacillus salivarius ABP118α	ABP118α	Gram-positive foodborne pathogen	Flynn et al. (2002)
Ruminococcus gnavus E1	Ruminococcin A	*C. perfringens*	Dabard et al. (2001)
R. gnavus E1	Ruminococcin C	*C. perfringens*	Crost et al. (2011)
Escherichia coli	Microcin C7	Pathogens like *Kleibsella*, *Salmonella*, *Shigella*, *Yersinia,* and *Proteus*	Garcia-Bustos et al. (1985)
E. coli	Microcin B17	Gram-negative bacterial species	Heddle et al. (2001)
E. coli	Microcin J25	Gram-negative bacterial species including *E. coli*	Mukhopadhyay et al. (2004)

(continued)

Table 19.1 (continued)

Host site and producer	Compound	Antagonistic activity against	References
E. coli	Microcin H47	Gram-negative strains including *E. coli*, *Salmonella*, *Enterobacter*, *Shigella*, *Klebsiella*, and *Proteus* spp.	Šmajs et al. (2010)
E. coli G3/10	Microcin S	Enteropathogenic *E. coli* E2348/69	Zschüttig et al. (2012)
E. coli	Colicin	Adherent-invasive *E. coli*, uropathogenic *E. coli*	Cohen et al. (2018)
Skin microbiome			
Staphylococcus epidermidis 5	Pep5	Staphylococci and Micrococci strains	Sahl and Brandis (1981)
S. epidermidis TU3298	Epidermin	Staphylococci and Streptococci strains	Allgaier et al. (1986)
S. epidermidis K7	Epilancin K7	Staphylococci and Micrococci strains	Van De Kamp et al. (1995)
S. epidermidis BN280	Epicidin 280	Staphylococci and Micrococci strains	Heidrich et al. (1998)
S. epidermidis 15 × 154	Epilancin15X	Gram-positive pathogens, methicillin-resistant *S. aureus,* and vancomycin-resistant Enterococci	Velásquez et al. (2011)
S. epidermidis 224	Epidermicin N101	Methicillin-resistant *S. aureus, Enterococci* and biofilm-producing *S. epidermidis* strain	Sandiford and Upton (2012)
Staphylococcus hominis	Sh-lantibiotics	*S. aureus*	McAuliffe et al. (2001)
Staphylococcus capitis TE8	Antimicrobial peptides	Gram-positive bacteria including *S. aureus*	Kumar et al. (2017)
Vaginal microbiome			
Lactobacillus gasseri	Lactocillin	Vaginal pathogens	Donia et al. (2014)

I_1 to I_9; I_1: *M. luteus*, I_2: *S. pyogenes* FF-22, I_3: *S. constellatus*, I_4: *S. uberis*, I_5: *S. pyogenes* 71-679, I_6: *L. lactis* T-21, I_7: *S. pyogenes* 71-679, I_8: *S. pyogenes* W-1, I_9: *S. dysgalactiae*

variants with different activity spectra through recombinations (Janek et al. 2016). Nukacin has been hypothesized to target peptidoglycan precursor lipid III (Islam et al. 2012). These antibiotics have been previously shown to be effective against nasal pathogens. The nasal commensal *Staphylococcus lugdunensis* produces a new type of lantibiotic, lugdunin, which inhibits the growth of various gram-positive pathogens, methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant enterococci. It is a nonribosomally synthesized thiazolidine containing cyclic peptide antibiotic, which is proposed to act by disruption of the cell membrane. It is not prone to cause drug resistance in the target strains making it a potential drug against facultative bacteria causing opportunistic infection (Zipperer et al. 2016).

19.3.2 Oral Microbiome

Human mouth has a rich microbial diversity, and constant competition of the microbial residents shapes its community. One of the common pathogens, *Streptococcus mutans*, responsible for causing dental caries, carves out its niche and prospers in the same way. It secretes a hybrid NRPS/PKS peptide, mutanobactin, that keeps the growth of commensals including *Streptococcus mitis* and pathogens like *Candida albicans* in check (Wu et al. 2010). Several mutanobactins including mutanobactins A-D are potent antifungal antibiotics that inhibit yeast to mycelial transition of *C. albicans* (Matthew Joyner et al. 2010; Wang et al. 2012) and may also have immunomodulatory effects (Zvanych et al. 2015). *S. mutans* also secretes other compounds including mutacins to inhibit gram-positive microorganisms (Merritt and Qi 2012).

Streptococcus salivarius is one of the dominant naturally occurring bacterial populations. It also produces an armory of lantibiotics including (a) Salivaricin A and its natural variants A3, A4, and A5, (b) Salivaricin A2 and B, (c) Salivaricin 9, and (d) Salivaricin G32, which differs from the *S. pyogenes* SA-FF22 propeptide by just one amino acid that acts against the colonization of *Streptococcus pyogenes* (Wescombe et al. 2011). Salivaricin A was first characterized lantibiotics from the *S. salivarius* 20P3 strain, which shows inhibitory activity against all 83 *S. pyogenes* strains and 9 standard indicator strains (I_1–I_9). Sal A2 and Sal B are the two lantibiotics produced from *S. salivarius* K12, which is used as an oral probiotic.

19.3.3 Gut Microbiome

The human gut is home to trillions of microbes that are involved in digestion of indigestible foods. The gut microbiome is also implicated in obesity, diabetes, autism, and irritable bowel diseases and can increase health-span of an individual (Sun and Chang 2014). The commensals produce a range of metabolites that act against pathogenic microbes. Gassericin, one of the initial bacteriocins reported from the human gut, is a cyclic lantibiotic and has specific activity against gram-positive foodborne pathogens. Out of 70 bacteriocin-producing *Lactobacillus* strains isolated from human feces, gassericin A from *Lactobacillus gasseri* LA39 was the most effective against the studied gram-positive pathogenic strains such as *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus* but had no activity against gram-negative bacteria (Kawai et al. 1994). Other cyclic lantibiotics include Reutricin and Circularin A from human gut isolates that possess different antimicrobial spectra. Reutricin has lytic activity against *L. delbrueckii*, inhibits growth of *B. cereus*, while Circularin A is effective against *Clostridium* spp. and has the most efficient activity against *C. tyrobutyricum* (a known cheese spoiler). The first bacteriocin reported from a probiotic, *Lactobacillus salivarius*, ABP118α is a two-component bacteriocin, which inhibits various foodborne pathogens like *Bacillus*, *Listeria*, *Enterococcus,* and *Staphylococcus* spp. and is heat stable (Flynn et al. 2002).

Ruminococcin A is a bacteriocin reported from the anaerobic bacterium *Ruminococcus gnavus* E1with a lanthanoin structure from the human gut. It is resistant to major host proteases and requires trypsin for its production. It is present in various fecal strains belonging to *Ruminococcus* sp. and *Clostridium coccoides* clusters in healthy adults and those suffering from chronic pouchitis and shows activity against *Clostridium perfringens* (Dabard et al. 2001; Marcille et al. 2002). *Ruminococcus gnavus* E1 also produces Ruminococcin C which has trypsin-dependent activity and similar antimicrobial spectrum to Ruminococcin A (Crost et al. 2011).

Gut enterobacteria also produce bacteriocins that act against the growth of pathogens. These bacteriocins may be either large-sized colicins (Cascales et al. 2007) or small-sized (<10 kDa) microcins (Garcia-Bustos et al. 1985) targeting different cell processes. Colicin V is one of the recent colicins described by screening a metagenomic library derived from the human gut microbes (Cohen et al. 2018). It is specifically active against *Escherichia coli* and may be used as therapeutic against adherent invasive *E. coli* (AIEC) associated with Crohn's disease or uropathogenic *E. coli* (UPEC) associated with urinary tract infections. Various microcins (Mcc) reported from *E. coli* usually have a narrow spectrum including MccS which prevents adherence of pathogenic *E. coli* to the gut (Zschüttig et al. 2012). Interestingly, MccC7 can inhibit many pathogenic species including pathogens belonging to *Klebsiella*, *Salmonella*, *Shigella*, *Yersinia*, and *Proteus* (Garcia-Bustos et al. 1985).

19.3.4 Skin Microbiome

Microbes residing on the skin utilize lipids, urea, and other components from sweat as nutrients to grow and in turn protect the skin from various skin pathologies like atopic dermatitis, psoriasis, etc. (Kong and Segre 2012; Sanford and Gallo 2013). They interact with the immune system and evade pathogens by maintaining a local pH and production of secondary metabolites and bacteriocins (Mousa et al. 2017). The discovery of Pep5 in 1981 from a human commensal *S. epidermis* strain 5, restricting against staphylococci and micrococci strains (Sahl and Brandis 1981), led to future discoveries from various skin commensals. Other Pep5-like bacteriocins produced by different strains of *S. epidermidis* include epidermin (Allgaier et al. 1986), epilancin K7 (Van De Kamp et al. 1995), and epicidin 280 (Heidrich et al. 1998). Epidermin exhibits low inhibitory activity against *Propionibacterium acne* and a number of staphylococcal and streptococcal strains (Allgaier et al. 1986). Epilancin 15X, which is active against several pathogenic bacteria, including methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci, and other staphylococci, is structurally similar to other lantibiotics described above.

Experiments proved that the N-terminal lactate group confers stability against proteolytic degradation by aminopeptidase. This feature may be applied for engineering of novel antibiotic with enhanced antimicrobial activity (Velásquez et al.

2011). Epidermicin N101, the unmodified bacteriocin produced by *S. epidermidis* strain 224, exhibits potent antimicrobial activity against MRSA, enterococci, and biofilm-producing *S. epidermidis* strain. It is active in nanomolar range with a high degree of protease stability and active over pH range of 2–10 (Sandiford and Upton 2012). A skin isolate, *Staphyloccocus capitis* TE8, was shown to possess antibacterial activity against gram-positive bacteria including *S. aureus*. The *Staphyloccocus capitis* TE8 genome possesses a repertoire of antimicrobial peptides including epidermicin, gallidermin, a gene cluster with phenol-soluble modulins (PSMβ 1-6), and a hypothetical protein, HPT2388. Synthetic peptides with sequences from PSMβ 1–6 and HPT2388 showed antibacterial activity (Kumar et al. 2017). In another study on the skin isolate *Staphylococcus hominis*, it was shown that *Sh*-lantibiotics can act independently or along with LL-37, a human antimicrobial peptide (AMP), to selectively kill *S. aureus*. In atopic dermatitis, *S. aureus* aggravates the disease; therefore, application of these lantibiotics and introduction of *S. epidermidis* and *S. hominis* on diseased skin have been shown to decrease *S. aureus* (Nakatsuji et al. 2017).

19.3.5 Vaginal Microbiome

The vaginal microbiome plays an important role in the prevention of urogenital diseases including bacterial vaginosis and candidiasis. Different *Lactobacillus* sp. protect the host from bacterial vaginosis by maintaining a low pH and H_2O_2 production, which prevents colonization by pathogens (Eschenbach et al. 1989). A new thiopeptide, lactocillin, from the vaginal commensal *Lactobacillus gasseri* was characterized to be active against most vaginal pathogens including *Gardenella vaginalis*, *Staphylococcus aureus*, *Cornyebacterium ahrimucosum*, and *Enterobacterium faecalis*, while the commensals were resistant to lactocillin (Donia et al. 2014). It is structurally different from the known thiopeptide antibiotics and can be manipulated to be used as drug in the clinic.

19.4 Microbiome and Anticancer Compounds

The presence of secondary metabolites produced by human commensals also stems proliferation of tumor cells. For example, 6-*N*-hydroxyaminopurine (6-HAP) produced by *Staphylococcus epidermidis* selectively targets tumor cells and may confer protection against skin neoplasia. 6-HAP is a nucleobase analog of adenine, in which the amino group of the C6 carbon is replaced with an *N*-hydroxyl group and inhibits binding of adenine with cytosine stalling replication in the process. The specific antiproliferative effect is mediated by low expression of mitochondrial amidoxime-reducing components (mARC1 and mARC2) in tumor cells. The inhibitory effect was observed in tumor cell lines and on injection or topical application of the metabolite or *S. epidermidis* whole cells in vivo in mice (Nakatsuji et al. 2018).

Microbially produced antibiotics and peptides are been considered as therapeutics for cancer treatment (Chakrabarty et al. 2014). Administration of purified azurin, a redox protein from *Pseudomonas aeruginosa*, could induce apoptosis via activation of p53 (Yamada et al. 2002). The prospect of human microbiome possessing azurin-like molecules was explored through a large-scale genomics analysis. Eight novel azurin-like peptides were identified, which may possess anticancer properties (Nguyen and Nguyen 2016).

The possibility of gut microbial metabolites mediating the killing of colorectal cancer cells can also be considered. Owing to the ability of a few gut commensals such as *Fusobacterium* spp. to hydrolyze gut tannin to gallic acid (GA) and pyrogallol (PG) using tannase, these microbes dominate in tumor cells where metastasis is promoted by tannin. GA and PG, on the other hand, exhibit cancer-killing properties by producing reactive oxygen species, which in turn activate p53 in these cells to induce apoptosis (de Felipe et al. 2014). These gut commensals can be utilized as potent drugs against colonic cancers.

19.5 Immunomodulatory Metabolites from the Human Microbiome

Small molecules from the human microbiome can also modulate the host immune response as a form of defense against the pathogens. These molecules are identified using a combination of genetics and biochemistry. For example, the BF2461 gene from *Bacteroides fragilis*, an abundant gut commensal with a sphingolipid-rich membrane, codes for a putative serine palmitoyl transferase, whose yeast homolog catalyzes the synthesis of sphingolipids including α-glycosylceramide: KRN7000. KRN7000 can serve as a ligand for CD1d-restricted natural killer T (iNKT) cells. This is the first member of a bacterial sphingolipid pathway identified from a human commensal. Sphingolipids are a class of signaling molecules that play a key role in inducing apoptosis and modulating the host immune response. When this lipid was introduced in mouse and human, it bound to CD1d and activated iNKT cells both in vitro and in vivo like KRN7000, and it might serve as a ligand for CD1d to influence host immune response (Wieland Brown et al. 2013). Similarly, other microbial molecules can also illicit immune responses. In a functional metagenomic study, screening of commensal bacterial effector genes (Cbegs) that activate the human transcription factor NF-κB was conducted. Cbegs can encode for molecules that can interact with receptors on human cells. NF-κB arbitrates response to environmental stimuli. In the screen, a Cbeg encoding a previously unreported molecule, *N*-acyl-3-hydroxypalmitoyl-glycine (commendamide), was identified from 26 unique effector genes. Commendamide could also activate the G-protein–coupled receptor (GPCR) G2A by mimicking the activity of endogenously produced N-acyl-amide signaling molecules that bind with the GPCR GPR132/G2A (Cohen et al. 2015). G2A has implications in autoimmune diseases and atherosclerosis, and thus, commendamide may promote these diseases.

Gut microbiota influence host behavior, but the mechanisms are not well studied. In a study, Williams et al. (2014) observed that decarboxylase (TrpD) of *Clostridium sporogenes* and *Ruminococcus gnavus* is capable of decarboxylating tryptophan to tryptamine. This enzymatic activity was previously found in plants and only a few bacterial soil isolates, that is, *Xenorhabdus nematophilus* and *Bacillus atrophaeus*. TrpD was found to be present in at least 10% of the studied human metagenomic samples. Dietary tryptophan is converted to serotonin, which influences behavior. These gut microbes may sequester the dietary tryptophan and convert it to tryptamine, thereby decreasing tryptophan available for conversion to serotonin and hence modulate human behavior (Williams et al. 2014).

Apart from eliciting immune response against invading pathogens, small molecules from human commensals may contribute to improvement of general health of an individual. In a notable study, a genetically tractable gut commensal, *Bacteroides thetaiotaomicron*, was engineered to produce tryptamine from food-derived tryptophan to test for improvement of constipation-like gut disorders. Gut symbionts including *Ruminococcus gnavus* and *Clostridium sporogenes* encode for tryptophan decarboxylase (TrpD), which converts tryptophan to tryptamine. TrpD gene from *R. gnavus* was integrated into the chromosome of *B. thetaiotaomicron*, and the engineered stain was introduced into germ-free mice. In mice, tryptamine generated in vivo was found to interact with the GPCR 5-HT_4R, which lead to cAMP activation and increased secretion of colonic fluids. These fluids caused whole-gut mobilization of food leading to relief from constipation-linked IBS symptoms (Bhattarai et al. 2018).

19.6 Conclusion and Future Prospects

Recent discoveries in the last few decades have improved our understanding of the human microbiome and its role in human health and diseases. The presence of a large number of biosynthetic gene clusters in metagenome or genomes of bacterial isolates from human microbiome and identification of novel bioactive compounds/ peptides have opened up a new avenue for discovering novel therapeutics. Engineering of human microbiome to sense and respond to metabolic signals/pathogens and to respond by producing chemicals or bioactive molecules in vivo is expected to revolutionize the healthcare in the future.

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