Vijay Tripathi · Pradeep Kumar
Pooja Tripathi · Amit Kishore
Madhu Kamle *Editors*

Microbial Genomics in Sustainable Agroecosystems

Volume 2

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Foreword

Microorganisms, or microbes, play a vital role in our lives and represent the richest diversity. The analysis of their functional diversity using omics approaches is very important for the sustainable agroecosystem, as the diversity of microorganisms is the indicator of the quality of agroecosystem. Exploiting microbes for multiple activities to benefit human life and sustainable agroecosystem is the need of the day. Advancement of omics approaches opens new vistas to unravel the genes, genomics, and mechanisms in various microbes using meta-genomics. The improved advancement of NGS based techniques, whole genome and transcriptome for exploiting the microbial biodiversity for sustainable agroecosystem.

This book of microbial genomics for sustainable agroecosystem reveals the tremendous potential of omics approaches in revealing the microbes as powerhouse from drug discovery to bioremediation. Omics studies hold tremendous potential to facilitate the screening of microbial secondary metabolites to plant growth-promoting microbiomes for crop improvement.

This edition of *Microbial Genomics for Sustainable Agroecosystem* (Volume II) is a commendable step in this area. I am extremely delighted to read this book as a researcher working in the area of microbial genomic and found its content to be informative, interesting, and updated. I express my sincere gratitude toward the editors of this book who made a significant contribution in conceptualizing this book which compiles a vast array of chapters starting from alkaline proteases, heavy metal tolerance, and functional genomics to bioremediation. In my opinion, this book will be a milestone in microbial genomics that benefits researchers, students, faculty, and academicians around the globe.

I congratulate Dr. Vijay Tripathi (SHUATS, Allahabad), Dr. Pradeep Kumar (NERIST, Arunachal Pradesh), Pooja Tripathi (SHUATS, Allahabad), Amit Kishore (KNPG College, India), and Dr. Madhu Kamle (NERIST, Arunachal Pradesh) for

this wonderful compilation and in-depth efforts made by the authors which unravel the multi-omics approaches of microbes. At last, I appreciate this valuable contribution made by the editors which unlocks the understanding of microbial ecosystem, expanding the horizon for finding out the multi-omics applications.

Thanks

ERA Chair, VALORTECH Vijai Kumar Gupta
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Preface

We have entered an era where there is an ecosystem degradation and climate change; thus, there is an extreme need to maximize microbial functions in agrosystems for the future of global agriculture. It is universally known that microbial ecology has a very massive diversity in the earth other than any group of organisms and bacteria that has numerous microbial groups in the agroecosystems that affect the agricultural and environmental health. In recent years, various novel technologies have been developed to access the various microbial genomes using next-generation sequencing, informatics, and so on. In this book we present the interdisciplinary research strategies to revamp microbiome functions in agroecosystems.

Microbial Genomics in Sustainable Agroecosystems: Volume II explores the application of different microbial genomic approaches in the development of sustainable agroecosystems. This volume has value-added collections of 17 diverse chapters spanning various aspects of microbial genomics and provides comprehensive and updated information on a wide variety of topics that focus on the application of different genomics techniques in microbial research, crop improvement, plant health, soil management, identification of novel antibiotic target, climate change research, and exploring microbial diversity and bioremediation of heavy metals. The microbial genomics approaches explore the impact of the microbial community on agriculture and improve the crop variety that is generally the basis of human nutrition.

The chapter "Shared microbiome in different ecosystems: a meta-omics perspective" explains recent progress in the sequencing technologies and other omics approaches that have had a profound impact on microbiology and helped to develop a more complete picture of the microbial composition and function of different ecosystems. This chapter also describes current meta-omics projects studying this issue and addresses the potential of publicly available data. Therefore, chapter "Application of molecular and sequencing techniques in analysis of microbial diversity in agroecosystem" describes the recent advances in molecular biology especially in the DNA sequencing technology to provide more opportunities for comprehensive studies of these multifaceted microbes in agroecosystems. The chapter "Bioinformatics Resources for Microbial Research in Biological Systems" describes the microbiome research works in different biological domains, microbial databases, and tools, which can be useful for the application of microbes in emerging applied fields. The chapter "Applications of microarray-based technologies in

identifying disease-associated single nucleotide variations" emphasizes the involvement of microarrays technologies and their applications in areas of cellular and molecular biology and their use for diagnostic and therapeutic purposes. The chapter "Impact of microbial genomics approaches for novel antibiotic target" describes an overview of the microbial genomics approaches such as pan-genomics, comparative genomics, functional genomics, structural genomics, transcriptomics, and proteomics used in the discovery and development of novel antibiotics. The chapter "Next Generation Sequencing (NGS) platforms: An Exciting Era of Genome Sequence Analysis" explains about the various DNA sequencing techniques and their application in different areas. In the chapter "Annotation of Biological Network of Fungus *Saccharomyces cerevisiae* Using Cytoscape in System Biology," the authors used unfasten basis software Cytoscape for integrating the biomolecular-interaction networks among elevated throughput appearance data and shaped circular arrangement of a cell recitation of all genetic interactions. The chapter "Recent Advances in Microbial Genome Sequencing" emphasizes the advancement of microbial genome sequencing technology in rapid microbial characterization, pathogen detection, and understating of microbial evolution. The chapter "Functional Genomics of Microbial Pathogen for Crop Improvement" explains the role of various functional genomics approaches in assessing the contribution of plant pathogens and microbial communities in the plant diseases.

The chapter "Role of microbial genomics in plant health protection and soil health maintenance" explains the main functions of rhizospheric microorganisms and their impact on plant and soil health maintenance. The chapter "Role of Microbial Genomics in Crop Improvement" explains the application of the microbial genomics method in crop production and crop improvement. The chapter "Current Status and Future Prospects of Omics Tools in Climate Change Research" summarizes various aspects of omics tools and their future scope that can be utilized in climate change research. The chapter "Plant and microbial genomics in crop improvement" discusses about the soil microbiology and molecular plant nutrition for sustainable food production and various omics tools to understand the living systems that exist in the soil and their interaction with the plants. The chapter "Alkaline protease: A tool to manage solid waste and its utility in the detergent industry" focuses on the utility of alkaline protease in management of solid waste and in the detergent formulation, and the method to improve the capability of a microorganism to increase the yield of alkaline protease as a source of animal feed. The chapter "Heavy metal toxicity and possible functional aspects of microbial diversity in heavy metal contaminated sites" describes the metal sources with their toxicity activities in the environment and discusses about different tools to understand the microbial diversity in heavy metal contaminated sites. The chapter "Bioremediation of nutrients and heavy metals from wastewater by microalgal cells: Mechanism and kinetics" focuses mainly on the primary mechanisms involved in the assimilation of nitrogen, carbon, and phosphorus inside the microalgal cell. Not only a brief description of metal-ion uptake by processes such as ion exchange, complex formation, precipitation, physical adsorption, and role of the plasma membrane, cell wall, vacuoles, chloroplast, and mitochondria is discussed in this

investigation but also the various kinetic models of nutrient removal such as Stover-Kincannon, Michaelis-Menten, Gompertz model, and Luedeking-Piret model with their experimental curve-fitting results obtained from the microalgal cell-mediated treatment process are discussed. The chapter "Meta-omics in the detection of silkworm gut microbiome diversity" focuses on the understanding of current knowledge on the investigation of insect gut microbes, especially in silkworms, and their functional role in the insect gut environment. Bridging the gap between the unknowns of silkworm gut microbiota by the appropriate tool such as metagenomics in combination with metaproteomics helps in this strategy.

This volume is the collaborative work of many people. We hope that this book will be beneficial for the undergraduate, postgraduate, research scholars, and researchers who are working in the expanding field of microbiology, environmental science, bioinformatics, and biotechnology.

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Contents

1 **Shared Microbiome in Different Ecosystems: A Meta-Omics Perspective** . . . 1
Arghavan Alisoltani, Akebe Luther King Abia, and Linda Bester

2 **Application of Molecular and Sequencing Techniques in Analysis of Microbial Diversity in Agroecosystem** . . . 21
Shobhika Parmar, Vijay Kumar Sharma, and Jitendra Kumar

3 **Bioinformatics Resources for Microbial Research in Biological Systems** . . . 45
Brijesh Singh Yadev, Pallavi Chauhan, and Sandeep Kushwaha

4 **Applications of Microarray-Based Technologies in Identifying Disease-Associated Single Nucleotide Variations** . . . 61
Sartaj Khurana, Sudeep Bose, and Dhruv Kumar

5 **Impact of Microbial Genomics Approaches for Novel Antibiotic Target** . . . 75
Hemant Joshi, Akanksha Verma, and Dharmendra Kumar Soni

6 **Next-Generation Sequencing (NGS) Platforms: An Exciting Era of Genome Sequence Analysis** . . . 89
B. Meera Krishna, Munawwar Ali Khan, and Shams Tabrez Khan

7 **Annotation of Biological Network of Fungus *Saccharomyces cerevisiae* Using Cytoscape in Systems Biology** . . . 111
Prashant Ankur Jain, Ved Kumar Mishra, and Satyam Khanna

8 **Recent Advances in Microbial Genome Sequencing** . . . 131
Rajpal Srivastav and Garima Suneja

9 **Functional Genomics of Microbial Pathogens for Crop Improvement** . . . 145
Neelam Chaudhary, Arun Kumar, and Baudh Bharti

10 **Role of Microbial Genomics in Plant Health Protection and Soil Health Maintenance** . . . 163
Arpna Ratnakar and Shikha

11 Role of Microbial Genomics in Crop Improvement 181
Mamta Gupta, Priya Chugh, and Arun Kumar

12 Current Status and Future Prospects of Omics Tools in Climate Change Research 197
Himashree Bora, Sukni Bui, Zeiwang Konyak, Madhu Kamle, Pooja Tripathi, Amit Kishore, Vijay Tripathi, and Pradeep Kumar

13 Plant and Microbial Genomics in Crop Improvement 215
Indu Rialch, Saurabh Singh, Rajender Singh, and Arun Kumar

14 Alkaline Protease: A Tool to Manage Solid Waste and Its Utility in Detergent Industry 231
Vipul Kumar Yadav, Veer Singh, and Vishal Mishra

15 Heavy Metal Toxicity and Possible Functional Aspects of Microbial Diversity in Heavy Metal-Contaminated Sites 255
Pradeep K. Shukla, Pragati Misra, Navodita Maurice, and Pramod W. Ramteke

16 Bioremediation of Nutrients and Heavy Metals from Wastewater by Microalgal Cells: Mechanism and Kinetics 319
Vishal Singh and Vishal Mishra

17 Meta-omics in Detection of Silkworm Gut Microbiome Diversity 359
Mohanraj Ponnusamy, Chinnan Velmurugan Karthikeyan, and Babu Ramanathan

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1 Shared Microbiome in Different Ecosystems: A Meta-Omics Perspective

Arghavan Alisoltani, Akebe Luther King Abia,
and Linda Bester

Abstract

Recent progress in the sequencing technologies and other omics approaches have had a profound impact on microbiology and helped to develop a more complete picture of the microbial composition and function of different ecosystems. One of the observations from meta-omics research is some microbes are ubiquitous in diverse ecosystems and that such shared microbiota could act as a backbone to support ecosystem function. This chapter describes current meta-omics projects studying this issue and addresses the potential of publicly available data to (i) identify the shared microbes that inhabit different environments and to (ii) study the microbial-core functions. We also discuss key challenges, gaps, and perspectives of meta-omic studies to help researchers to take the next steps forward.

Keywords

Core function · Meta-omics · Microbiota · Shared microbiome

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1.1 Introduction

The term meta-omics refers to the unbiased study of biomolecules (DNA, RNA, protein, and metabolites) directly recovered from environmental ecosystems. Meta-omics have provided an excellent opportunity for the identification of the diversity, frequency, and composition as well as the function of microbial communities. Characterization of the composition and function of microbiota can reduce the complexity of various ecosystems to better understand the adaptation, dynamics, and evolution of microbial communities. Recent interests in meta-omics studies can be attributed to the advances in omics technologies, especially high-throughput sequencing techniques.

To estimate the rate of meta-omics studies, we have targeted the Sequence Read Archive of the National Center for Biotechnology Information (SRA-NCBI), MGnify, Joint Genome Institute (JGI), and Google Scholar. A sharp increase in the available data in the SRA-NCBI is observed since 2013 (Fig. 1.1a). Results of literature review and records of SRA-NCBI, JGI, and MGnify (EMBL-EBI Metagenomics) show that metagenomic studies are much more prevalent than the

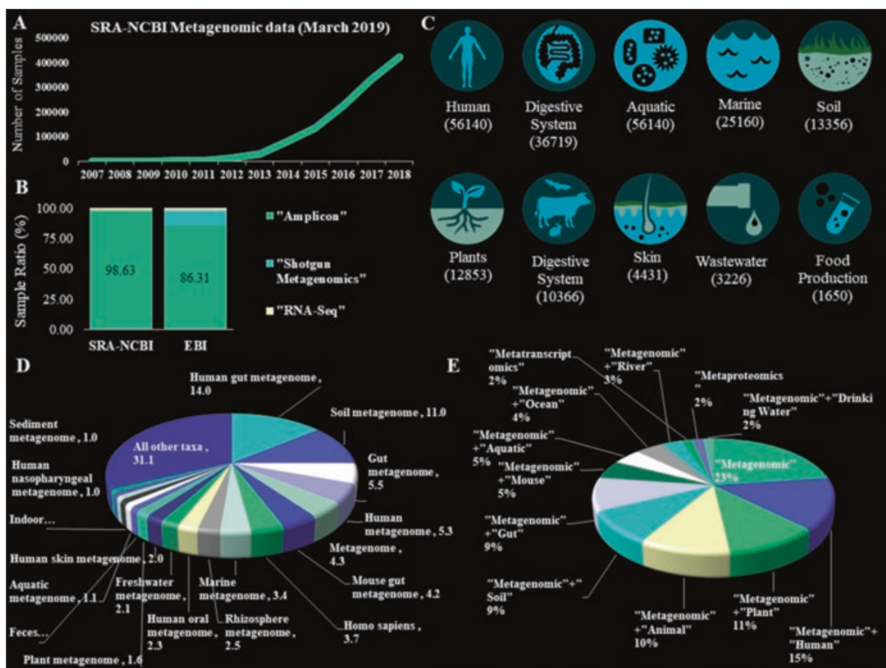


Fig. 1.1 General statistics of meta-omic studies as of March 2019. (**a**) Trend of data growth in the SRA-NCBI; (**b**) The ratio of metagenomics and metatranscriptomic studies in SRA-NCBI and MGnify (EMBL-EBI); (**c**) Number of available data in MGnify considering the type of sample; (**d**) Ratio of available data in SRA-NCBI based on the type of sample; (**e**) Meta-omic publication ratio reflecting Google Scholar searches

metatranscriptomic studies (Fig. 1.1b), probably due to the difficulties of extracting RNA, higher sequencing cost, and complexity of data analysis. The MGnify statistics also indicate that the majority of meta-omics studies have focused on the human, particularly the digestive, system (Fig. 1.1c). Exploring SRA data and Google Scholar publications showed a similar trend (Fig. 1.1d, e), suggesting more attention to other types of species and habitats is required as well. In general, the human digestive system, marine, soil, plant, and animal digestive systems are among the most studied ecosystems, respectively (Fig. 1.1).

The above statistics indicate that meta-omic analysis has become a routine practice to study the composition and function of microbes in different ecosystems. A majority of the microbial populations are considered unculturable, and one of the advantages of metagenomics is capturing nearly 90% of the microbial population using the culture-independent approaches. Although a high level of diversity and complexity of microbial population has been identified using metagenomic studies, some of the operational taxonomic units (OTUs) are reported as a commonly occurring microbiota across a particular or different ecosystem (Bolourian and Mojtahedi 2018b; Samad et al. 2017; Selden et al. 2016). However, the level of the uniqueness and commonness of OTUs is varied based on the type of ecosystem. For instance, the human gut is said to have a lower number of shared OTUs compared to the intraoral niches, although the trend might not be similar for other types of species. Anwis et al. identified both unique and shared gut microbiome species in Welsh Mountain ponies. They reported that the unique structure and social interaction of the population influenced the microbial composition (Antwis et al. 2018). Using 16S metagenomic analysis, it was identified that river sediment samples had a similar bacterial composition different from those recovered from river water (Abia et al. 2018). A series of recent research results have indicated that the microbes from the natural environment can be protective against diseases such as allergy and autoimmunity (Bolourian and Mojtahedi 2018a; Flandroy et al. 2018). For instance, Bolurian and Mojtahedi (2018b) hypothesized that lack of *Streptomyces*, a shared soil and gut bacterial genus, could contribute to colon cancer development in human.

In general, exploring the shared microbiome is important to (i) identify a core microbiome of each condition; (ii) understand the microbial-functional interrelations; (iii) distinguish an early diagnosis of shifting from normal conditions; and (iv) detect ubiquitous-pathogenic taxa. In the next sections, we describe the commonly identified microbiome across different habitats by focusing on publicly available meta-omic datasets and recently published studies.

1.2 What Are the Common Microbial Taxa in Different Ecosystems?

In this chapter, we analyze the MGnify database to obtain a holistic overview of the shared microbiome in different habitats. MGnify is a freely available resource for various types of microbiome derived sequence data. As of March 2019, a total of 2535 studies, 154,122 samples, and 207,351 analyses had been recorded in MGnify.

Table 1.1 Details of available metagenomics data retrieved from MGnify, including accession ID, type of sample, and the number of samples per study

Accession ID	Sample type	Number of samples per study
MGYS00003194	Aquatic – marine water	4427
MGYS00003961	Aquatic – lake water	1143
MGYS00000991	Aquatic – arctic ocean water	206
MGYS00003809	Sediment – marine sediment	276
MGYS00003914	Sediment – bay sediment	26
MGYS00003655	Sediment – Baltic marine sediment	10
MGYS00003922	Soil – barley soil	295
MGYS00001864	Soil – forest soil	841
MGYS00003917	Soil – farm soil	78
MGYS00001061	Soil – oil-contaminated soil	1
MGYS00000818	Soil – hydrocarbon polluted soil	1
MGYS00002019	Human – infant gut	294
MGYS00003481	Human – gut metagenome and metatranscriptome	1150
MGYS00003468	Human – digestive system	396

This database provides both raw and analyzed sequencing datasets and gives insights about microbial composition, diversity, and function of environmental samples. The primary criteria considered to select the datasets for each environment were the number of samples per dataset and the availability of the taxonomic and functional analysis results (Table 1.1). We focused on five main biomes in the MGnify database, including animal and human digestive systems, aquatic, sediment, and soil samples. The relative abundance of the microbial composition and function for each sample (MGnify results) were aggregated to identify the general composition of different ecosystems. In the following sections, we mainly discuss the dynamic changes of Archaea and *Bacteria* in the samples above. The term microbiome in this chapter refers to the entire microbes, active or inactive, exhibited in a specific sample and/or habitat. It should be noted that the selected samples and database might not be representative of the actual microbial diversity and composition.

1.2.1 Aquatic and Sediment Samples

The integration of MGnify results for the selected aquatic samples (Table 1.1) indicates that most of the marine sequencing data were overwhelmingly assigned to *Bacteria* followed by Eukaryota and Archaea (Fig. 1.2a). A similar trend was also observed for sediment samples (Fig. 1.2b) and other environments (Brown et al. 2015; Reese et al. 2018). For instance, in the metagenomic analysis of James River samples in North America, almost 97%, 2%, and 0.3% of the reads were assigned to *Bacteria*, Eukaryota, and Archaea, respectively (Brown et al. 2015). It could be either due to the more abundance of bacteria in the environment, or the methods are biased in terms of capturing the actual microbial population. Regardless of the

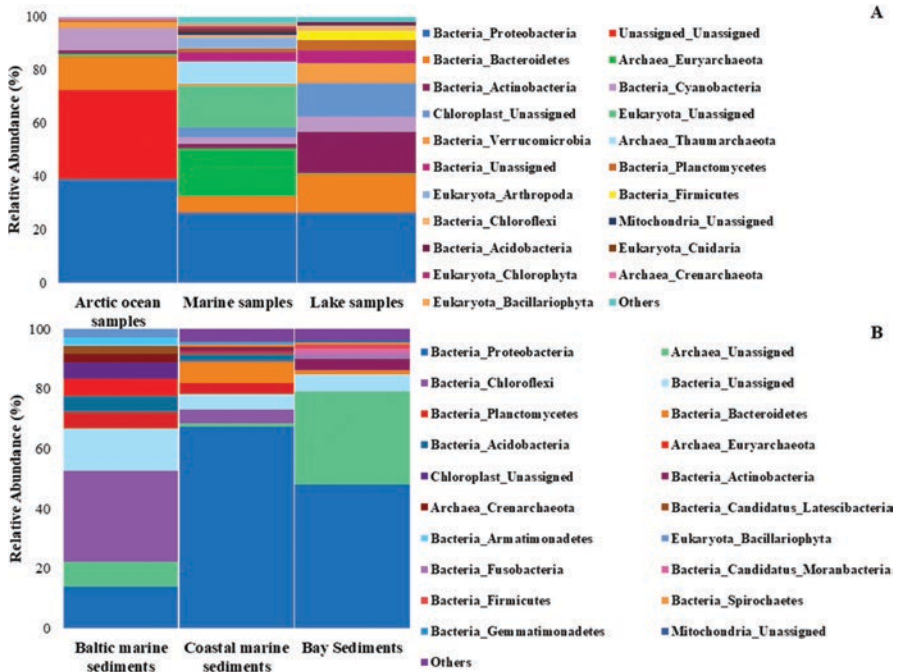


Fig. 1.2 The integrated relative abundance of top phyla in the selected samples obtained from MGnify (March 2019). (**a**, **b**) panels are representing the results of aquatic and sediment samples, respectively

unassigned (unknown) microbiome in the studied samples, *Proteobacteria* (a bacterial phylum) and three archaeal phyla Arthropoda, Euryarchaeota, and Thaumarchaeota were among the most abundant taxa identified from the aquatic ecosystems (Fig. 1.2a). The predominance of *Proteobacteria* is consistent with other reports on river water and sediment samples (Abia et al. 2018; Jordaan and Bezuidenhout 2016; Reza et al. 2018), water reclamation plant (Sekar et al. 2014), roof-harvested rainwater (Chidamba and Korsten 2015; Kirs et al. 2017), mine waters (Kamika et al. 2016; Keshri et al. 2015), and drinking water systems (Bautista-de los Santos et al. 2016; Saleem et al. 2018), indicating that members of these phyla are ubiquitous and able to survive in a wide range of environments.

The phylum Thaumarchaeota, the most abundant common archaeal group (Fig. 1.2a), is known as one of the dominant archaeal phyla on earth (Müller et al. 2018). Reji et al. demonstrated a depth-associated diversity pattern of this phylum in coastal ocean waters. They also reported that co-occurrence of Thaumarchaeota and different heterotrophic *Bacteria* such as *Gammaproteobacteria* might contribute to the diversification of Thaumarchaeota ecotypes (Reji et al. 2019). In addition to Thaumarchaeota, numerous archaeal species are recognized to inhabit different ecosystems, particularly extreme environments, including systems with high salinity, extreme temperatures, and high pressure as well as acidic systems (Singh et al.

2019). For instance, the phylum Euryarchaeota has been identified in several saline aquatic systems (Fig. 1.2a), and the elevated abundance of Euryarchaeota-affiliated species with the increase in salinity was demonstrated. Another example is the genus *Picrophilus*, a member of Euryarchaeota, containing species that can grow in ecosystems with very low pH (Quatrini and Johnson 2018).

Since the quality of drinking water (DW) is a critical public health issue, several studies have evaluated the presence of pathogenic microbes in aquatic systems. As shown in Table 1.2, *Mycobacterium*, followed by *Pseudomonas*, *Legionella*, and *Vibrio* are among the most abundant pathogenic genera across selected aquatic samples, respectively. Species of *Mycobacterium* are known as common inhabitants of DW biofilms, and some of them, such as *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Mycobacterium gordonae*, cause respiratory diseases (Basak et al. 2015; Chao et al. 2015; Revetta et al. 2016; Richards et al. 2015). In terms of sediment samples, *Pseudomonas* was the predominant pathogenic genus. This genus is considered as a biofilm developer and can increase the surviving ability of pathogens in aquatic systems (Navarro-Noya et al. 2013). Strains of *Legionella* have been reported as ubiquitous species because they can be found in different aquatic environments such as hot water systems (Farhat et al. 2012), roof-harvested rainwater (Chidamba and Korsten 2015), river water and sediments (Basak et al. 2015; Chidamba and Korsten 2015; Suriya et al. 2017), surface waters (Hsu et al. 2015), and DW biofilms (Lin et al. 2013). In addition to the mentioned microbes, seafood-borne microbial pathogens such as *Enterococcus faecalis* and *Enterococcus faecium* are said to have multidrug resistance which can lead to health-related problems (Jahan et al. 2015; Zhou et al. 2019).

The main factors that contribute to the diversification of microbial populations in water and sediments are pH, temperature, depth, nutrients, and contaminants as well as the presence of other microbes (Johnson et al. 2015; Mirete et al. 2016; Reji et al. 2019). Abia et al. (2018) reported that river sediment *Bacteria* were more diverse than the water column *Bacteria* in the Apies River, South Africa, probably due to the availability of more nutrients in the sediments compared to the water column. In the drinking water distribution systems (DWDSs), plumbing materials (e.g., uPVC and Cu coupon) can influence the microbial composition of drinking water (Buse et al. 2014). Revetta et al. (2016) suggested that the source of water and the water treatment processes also had a significant role in the structural variation of bacterial communities in DWDSs. However, microbial communities that live in the form of biofilms are resistant to water treatments with chlorine and other antibiotics than the ones in the planktonic forms (Lechevallier et al. 1988; Wingender and Flemming 2011; Xi et al. 2009; Yu et al. 2010). Microbes inhabiting biofilms are known to be challenging to treat and can negatively influence the color, taste, and odor of drinking water, which may threaten human health (Långmark et al. 2005; Prest et al. 2014). In the study of Ma et al., *Aspergillus*, *Candida*, and *Fusarium* were identified as potential pathogenic fungi in hospital hot water system. The authors declared that monochloramine water treatment did not influence the composition of fungi when compared to untreated water (Ma et al. 2015). These naturally occurring microbial biofilms were described as microbial reservoirs for further water contamination.

Table 1.2 The integrated relative abundance of common pathogenic bacteria in five main biomes of MGnify

Genus	Aquatic samples	Sediment samples	Forest and farm soil samples	Animal digestive system	Human digestive system
Bacillus	0.02	0.00	0.30	0.04	0.01
Bacteroides	0.03	0.00	0.09	1.45	1.65
Bartonella	0.00	0.00	0.00	0.00	0.00
Campylobacter	0.00	0.01	0.00	0.02	0.41
Corynebacterium	0.00	0.03	0.01	0.01	0.04
Escherichia	0.00	0.00	0.00	0.00	0.11
Haemophilus	0.00	0.03	0.00	0.00	0.22
Klebsiella	0.00	0.00	0.00	0.00	1.08
Legionella	0.07	0.00	0.01	0.00	0.00
Mycobacterium	0.12	0.00	0.07	0.00	0.00
Neisseria	0.00	0.11	0.00	0.03	0.21
Nocardia	0.00	0.00	0.00	0.00	0.02
Pseudomonas	0.11	3.96	0.28	0.18	0.01
Salmonella	0.00	0.00	0.00	0.00	0.00
Staphylococcus	0.00	0.01	0.01	0.08	5.57
Streptococcus	0.01	0.13	0.01	1.54	2.54
Treponema	0.00	0.00	0.00	0.08	0.00
Vibrio	0.03	0.00	0.00	0.00	0.00

1.2.2 Soil Samples

The composition and function of microbial communities in soil can be affected by a variety of factors such as soil pH, water content, depth, temperature, amount of organic matter, pollutants, and soil nutrients as well as the presence of competing species (Abia et al. 2019; Bell et al. 2013a, b; Meidute et al. 2008; Violle et al. 2011). The integrated MGnify results of three different soil types indicated the predominance of *Proteobacteria* followed by *Acidobacteria* and *Actinobacteria* (Fig. 1.3a), although a considerable amount of unassigned OTUs was also detected. It has been shown that *Actinobacteria* are abundant in soils with low organic matter, while on the contrary, *Proteobacteria* is the dominant phylum in soils with high organic matter (Bell et al. 2013b). *Bacillus*, *Pseudomonas*, *Bacteroides*, *Mycobacterium*, *Streptococcus*, and *Legionella* were recorded as the most abundant pathogenic bacteria in soil samples (Table 1.2). Interestingly, the mentioned pathogenic bacteria are common genera in the aquatic, sediment, and soil samples, although the frequency of these genera is varied (Table 1.2).

In addition to pathogenic bacteria, it has been found that *Chitinophaga* and *Nitrospira* are among common and dominant bacterial genera in many vegetated soils such as rice, maize, and tomato rhizospheres (Chung et al. 2012; Lee et al. 2016; Li et al. 2014; Yang et al. 2017). Some studies demonstrated that vegetation impacts the ability of microbial communities to degrade organic contaminants in soil. For instance, the study of Leewis et al. (2016) proved that a willow native to Alaska could accelerate the degradation of diesel contaminants. The authors concluded that willow, together with fertilizers, increased aromatic degradation by

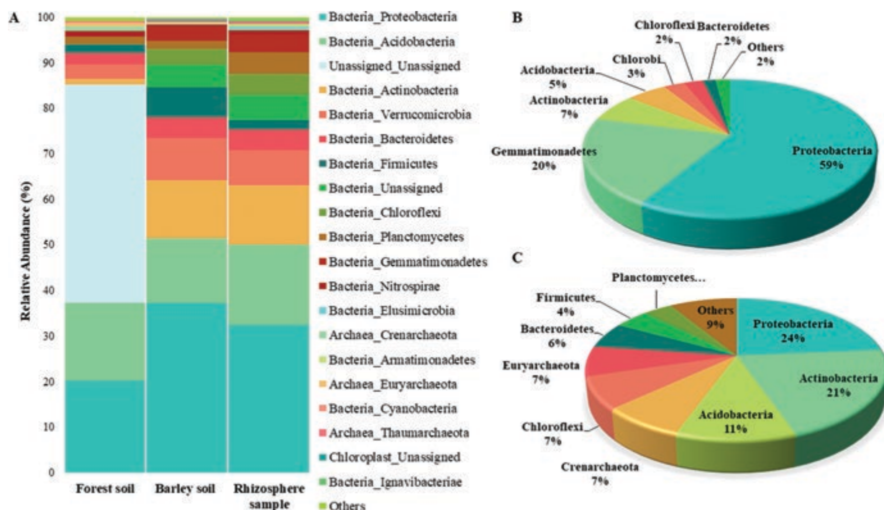


Fig. 1.3 The integrated relative abundance of top phyla in the selected soil samples obtained from MGnify (March 2019). (**a**) illustrates the results of forest and farm soils. (**b**, **c**) panels are representing the results of oil-contaminated and hydrocarbon-polluted soils, respectively

shifting the microbial community composition (Leewis et al. 2016). MGnify data indicates that *Proteobacteria* is a predominant phylum followed by *Acinetobacter* in hydrocarbon contaminated soils, as exemplified in Fig. 1.3b, c. Elevated abundance of *Proteobacteria* has been demonstrated along with increasing concentrations of petroleum hydrocarbons (Shahi et al. 2016), implying that strains of this phylum could play a pivotal role in hydrocarbon degradation. A well-known example is *Gammaproteobacteria* which has been reported as the class that includes the genera with known oil degraders such as *Acinetobacter*, *Alcanivorax*, *Marinobacter*, and *Pseudomonas* (Kostka et al. 2011). Some strains of *Acinetobacter* and *Stenotrophomonas* have been reported to be tolerant to high concentrations of heavy metals (Ramadass et al. 2016), suggesting the dual potential of these species for the degradation of both hydrocarbon and heavy metal contaminants.

As was expected, Archaea constitute a smaller fraction of the identified taxa when compared to *Bacteria*. Crenarchaeota, Euryarchaeota, and Thaumarchaeota were characterized as the most abundant phyla in the soil samples (Fig. 1.3a). The co-occurrence and dominance of these archaeal communities have been manifested in many diverse environments (Figs. 1.2 and 1.3), suggesting that a specific adaptation may elevate the ability of these archaeal phyla to survive in different environmental conditions. Crenarchaeota-affiliated species are involved in the nitrogen cycle in the soil, and they can assimilate a broad range of organic carbon materials (Francis et al. 2007; Seyler et al. 2014). Members of Thaumarchaeota and Euryarchaeota are thought to have a high capability to grow in heavy metal-rich environments (Zhang et al. 2017). The authors also identified the coexistence of methane production and iron reduction in soil-archaeal populations and assumed that some levels of ecological interactions must have occurred between methane-producing and iron-reducing strains in Euryarchaeota. A positive correlation between ammonia and the abundance of Euryarchaeota and Crenarchaeota has been reported (Reese et al. 2018), which might be a piece of evidence for the metabolic interrelations of the species in these phyla.

1.2.3 Human and Animal Microbiome

The microbial patterns of humans and animals (vertebrates) are considerably different from those described in aquatic and terrestrial environments as shown in Fig. 1.4. *Firmicutes* and *Bacteroidetes* were listed as the common dominant phylum in both human and animal digestive systems, as also described in the literature, inferring that gut-core microbiome might have remained stable during evolution across species. It is demonstrated that the microbial diversity is higher between niches (e.g., gut, oral cavity, and skin) within the same person when compared to the level of microbial variation in the population for a defined habitat such as the digestive system (Huttenhower et al. 2012). Two classes, *Clostridia* (*Firmicutes*) and *Bacteroidia* (*Bacteroidetes*), are known to play a vital role in plant fiber degradation in many ruminants (Cunha et al. 2011). The members of both *Firmicutes* and *Bacteroidetes* are proved as the core-human microbiota which have been extensively studied in the

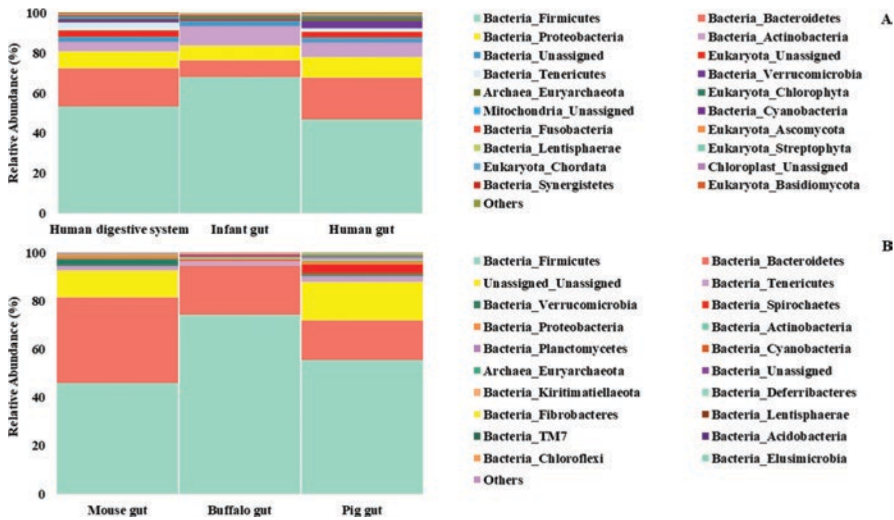


Fig. 1.4 The integrated relative abundance of top phyla in the selected digestive system samples obtained from MGnify (March 2019). (**a**, **b**) panels are representing the results of human and animal samples, respectively

past, such as the core gut microbiome in obese and lean twins (Turnbaugh et al. 2009), oral cavity (Zaura et al. 2009), and other body sites (Huse et al. 2012).

According to MGnify data, the most abundant OTUs shared across both human and selected animal gut samples belonged to seven genera including *Enterorhabdus*, *Ruminococcus*, *Staphylococcus*, *Prevotella*, *Oscillospira*, *Roseburia*, and *Streptococcus*. Most of these genera, including *Prevotella*, *Butyrivibrio*, *Lactobacillus*, *Streptococcus*, and *Ruminococcus*, are also recognized as the dominant core-rumen microbiome of several other animals regardless of geographical distribution (Henderson et al. 2015; Wang et al. 2016; Wirth et al. 2018). Wang et al. stated that *Atopobium*, *Quinella*, *Prevotella*, *Fretibacterium*, *and Ruminococcus* dominated the bacterial microbiota in goats with varied ages. The predominance of some of these genera is also reported in other parts of the human body such as *Staphylococcus* of the skin (Grice et al. 2009), *Streptococcus* in the oral cavity (Huttenhower et al. 2012), and *Prevotella* in females with bacterial vaginosis (BV) (Lennard et al. 2018; Mitchell et al. 2015). This is apparently because of the microbial trading and/or ability of these taxa to develop in diverse niches.

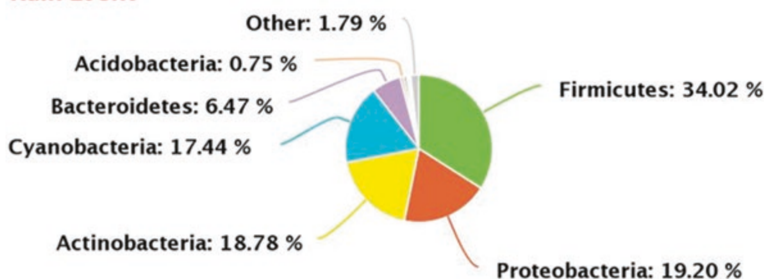
In terms of common pathogenic genera, *Bacteroides*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, and *Treponema* were recorded as the top pathogenic genera in the animal digestive system. Almost like that of animals, human samples were largely dominated by *Bacteroides*, *Klebsiella*, *Staphylococcus*, and *Streptococcus* (Table 1.2). Some of these genera are categorized as both noninvasive (commensal) and invasive (pathogenic/virulent) bacteria. The shifting from virulent to commensal status could be affected by the interrelation of different species. A well-known example is *Staphylococcus aureus* which can change from being

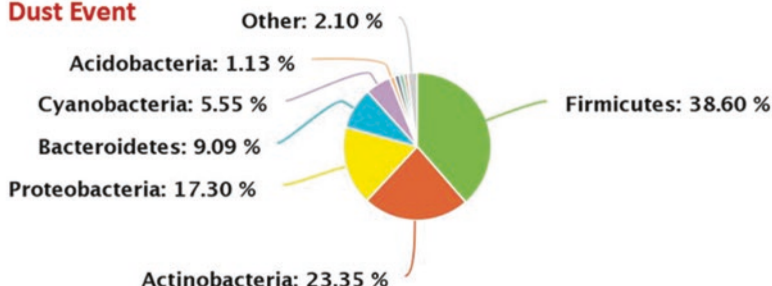
invasive to noninvasive in the presence of *Corynebacterium striatum* (Ramsey et al. 2016). Another example of interspecies interaction is the role of *Corynebacterium* spp. in preventing the growth and colonization of *Streptococcus pneumoniae* (Bomar et al. 2016).

The dynamic change in the archaeal community is less studied in many ecosystems when compared to that of *Bacteria*. Raymann and colleagues explained that about 90% of the archaeal composition and diversity of the gut microbiota had been largely ignored in the past, due to the application of classic *16 s rRNA* gene primers. The authors characterized 14 bacterium-archaeon associations that were shared across human and great ape species, such as the correlation of *Methanomassiliicoccales* with *Clostridiales* and *Mollicutes-RF39* (Raymann et al. 2017). MGnify data indicate that Euryarchaeota is the predominant phylum in both human and animal digestive systems broadly distributed in two methanogenic genera, including *Methanomassiliicoccus* (human) and *Methanobrevibacter* (animals). These methanogenic archaea are known to inhabit humans and other animal digestive systems (de Macario and Macario 2018; Nkamga et al. 2017). Methanogenic archaea are also seen in various ecosystems, including aquatic, sediment, and soil samples (Bendia et al. 2018; Besaury et al. 2014; Deng et al. 2019). In humans, methanogenic archaea such as *Methanomassiliicoccus* were detected in different body sites, such as the intestinal mucosa (Oxley et al. 2010), the oral cavity (Huynh et al. 2015), and skin (Moissl-Eichinger et al. 2017).

1.2.4 Atmosphere Microbiota

The atmosphere's microbiome has received less attention in comparison with other environmental ecosystems. Studies revealed that microbial composition and diversity of the atmosphere has a specific pattern which can be affected by several factors such as season, UV intensity, temperature, precipitation events, and humidity (Smets et al. 2016). Members of *Firmicutes* were considered the most abundant bacteria during dust events, whereas the proportion of *Proteobacteria* OTUs was higher in days with no dust event (Jeon et al. 2011). To epitomize, the bacterial composition of three MGnify samples obtained from rain, dust, and low dust events were depicted in Fig. 1.5. OTUs associated with *Firmicutes* were the most abundant taxa in all events especially during dust events, likely because of their immense capacity to survive harsh environmental conditions (Fig. 1.5). In the study of Polymenakou et al., it is explicated that the clone-associated *Firmicutes* dominated large bioaerosol particles, while *Actinobacteria* and *Bacteroidetes* were more abundant in the particles with reduced sizes. The authors pointed out that a significant number of clones found at the size of respiratory particles could be human pathogenic bacteria and were associated with various diseases (Polymenakou 2012; Polymenakou et al. 2007). However, the sampling method might influence the estimation of the taxonomic composition of the atmosphere as exemplified by Serrano-Silva et al. (2018).





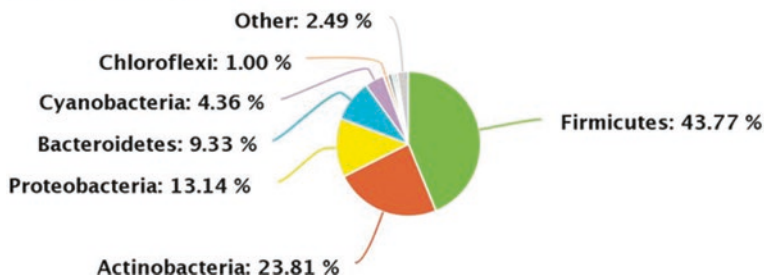


Fig. 1.5 The relative abundance of top phyla in three atmospheric samples obtained from MGnify (March 2019)

1.3 Beyond Microbial Composition and Diversity of Ecosystems

A step beyond determining the core-microbial composition of the ecosystems is the investigation of the function of shared microbiota. Two main factors might contribute to the co-occurrence and ubiquitous nature of microbes: first is the niche-overlap due to the shared environmental preferences, and second are the metabolic interactions for nutrient trading. Although numerous studies have endeavored to determine

the core-microbial composition of ecological systems, the core-function of these systems is not fully understood. In 2009, Turnbaugh and colleagues discussed that instead of inferring based on the core-microbial composition in the human gut, it could be more reliable and accurate to consider the core-function of the microbiota. The authors demonstrated that the level of functional diversity in the human gut was profoundly associated with the relative abundance of OTUs associated with *Bacteroidetes*, while a moderate functional variation was detected for *Firmicutes* and *Actinobacteria* (Turnbaugh et al. 2009), implying that core-microbial function presumably has remained conserved across species which are phylogenetically related.

Due to the complexity of the metagenomes, linking a specific metabolic process to a particular group of microbes is challenging. Therefore, researchers are employing various strategies to overcome this obstacle such as considering the life history of microbiota and/or shared functional attributes. MGnify data reflected that marine and sediment metagenomes were principally involved in biological and environmental processes, such as biosynthesis, metabolism, oxidation-reduction processes, photosynthesis, response to wounding, and transport (Fig. 1.6a, b). Haggerty et al. divided the marine bacteria into distinct groups according to their shared trophic attributes. They noted that photosynthetic activity was a well-known core-function of the phototroph group (e.g., *Prochlorococcaceae* and *Chroococcaceae*), whereas oligotrophic bacteria (e.g., *Flavobacteriaceae* and *Rhodobacteraceae*) co-occurred with nitrogen metabolism, stress response, and amino acids and derivatives (Haggerty and Dinsdale 2017). Several studies that targeted archaeal core-functions in marine revealed the presence of almost the same functions, including biosynthesis, degradation, oxidation-reduction activities (Chen et al. 2019; Louca et al. 2016; Tully 2019). The co-occurrences and associations of various bacterial and archaeal members have been reported in marine environments, suggesting a possible symbiotic and functional interrelation between these communities (Steele et al. 2011).

Notwithstanding the variation in the core-microbial compositions, a relatively similar core-function was observed across the soil and animal samples (Fig. 1.6c, d), apparently due to the higher interaction of animals with soil (Bolourian and Mojtahedi 2018b). It should, however, be noted that the relative occurrence of the pathways varied in each environment and sample (Fig. 1.6). An exciting observation was the OTUs involved in "viral entry into host cell" (GO:0046718), which is probably linked to bacterial defense (Rojas et al. 2017), or the role of viruses in the horizontal gene transfer. Since proteins of both host cells and viruses are involved in viral entry into the host cell (Raman et al. 2016), the mentioned GO term can be applied to annotate both the viral and host proteins (Foulger et al. 2015). Nacke et al. performed a metatranscriptome analysis to gain insights into the microbial composition and function of forest and grassland soils. They found the predominance of bacterial sequences compared to the other domains of life. In addition to GOs related to viral entry into the host cell, the authors also identified OTUs involved in the degradation of aromatic ring-containing pollutants, wood breakdown, and photosynthesis (Nacke et al. 2014).

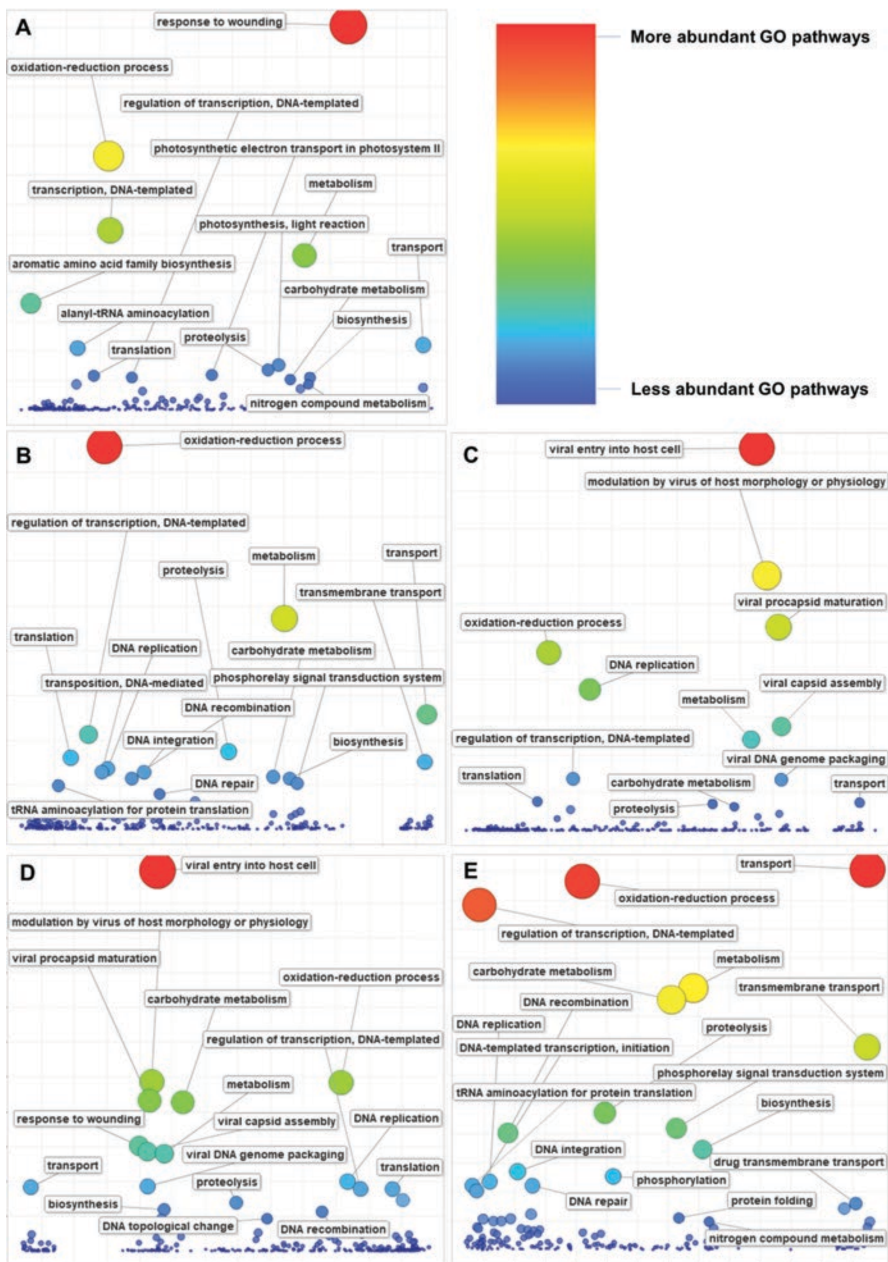


Fig. 1.6 Top GO biological processes constructed using REVIGO web tool based on targeted MGnify datasets in Table 1.1 (March 2019). (**a**–**e**) represent biological processes related to aquatic, sediment, soil, animal, and human samples

OTUs with the capacity to degrade the aromatic pollutants are critical for soil bioremediation. Zhang and colleagues (2010) discovered various bacterial strains that could degrade the petroleum hydrocarbons of diesel oil in Daqing, China. In another study, the impact of long-term diesel contamination on soil microbial community structure was investigated by pyrosequencing of the *16S rRNA* gene. The authors characterized several OTUs exhibiting a high level of similarity to anaerobic microbes known to be involved in petroleum hydrocarbon bioremediation, including members of the phyla *Chloroflexi*, *Firmicutes*, and Euryarchaeota (Sutton et al. 2013). Functional genes, mostly belonging to *Actinobacteria*, have been identified in the metabolism of aliphatic and aromatic hydrocarbons and resistance to heavy metals (Abbasian et al. 2016). Moreover, metabolic pathways of hydrocarbon-degrading bacteria from the Deepwater Horizon oil spill were reconstructed, and the researchers identified that the uncultured *Alphaproteobacteria* and *Gammaproteobacteria* populations were enriched in the polycyclic-aromatic-hydrocarbon-degrading communities and contained a broad range of gene sets for biodegradation of phenanthrene and naphthalene (Dombrowski et al. 2016).

The overall dominant functional pathways of microbial communities in the human digestive system were recorded as biosynthesis, transport, oxidation-reduction, metabolism, carbohydrate metabolism, proteolysis, and regulation of transcription as illustrated in Fig. 1.6e. The core-microbial structure and function of different human body sites have been extensively investigated in the past few years. By metatranscriptomic analysis of human gut microbiota, Gosalbes et al. identified a shared set of GO biological processes across all healthy individuals, and these included biogenesis, carbohydrate metabolism, translation, transport, and energy production. They also found that other less abundant GO terms such as lipid transport and metabolism were only common across some of the samples (Gosalbes et al. 2011). A higher level of microbial diversity in each individual was demonstrated in the human gut when compared to the level of functional variations (Turnbaugh et al. 2009), reflecting that these OTUs had similar functional activities, and thus could be substituted by each other to increase the stability and resilience of the human gut microbiota (Lozupone et al. 2012). Another well-studied shared function of the human gut microbiome is the resistance to antibiotics, in particular, the tetracyclines which has been characterized in 99% of individuals (Hu et al. 2013; Kaminski et al. 2015).

1.4 Conclusions and Perspectives

The knowledge of microbial composition and function in different ecosystems has rapidly grown due to the remarkable progress in the meta-omics approaches. In this chapter, we reviewed current meta-omics projects and MGnify data to obtain global insights into the shared microbiota inhabiting different ecosystems and their core functions. It is shown that a considerably large number of studies directed their research on microbial DNA than RNA, protein, and metabolites which can severely limit our understanding of the dynamic microbial function, especially information about live/active colonies and dead transient microbiota. Another challenge of metagenomic studies is the currently available metagenome references that can lead to potentially biased inferences,

hinting that there is a need for the more complete references and de novo sequence assembly to capture entire microbial taxa. Besides, probing the literature indicates that due to the complexity of the environmental metagenomes, inferences of function for individual species is challenging, particularly for the unknown/uncultured OTUs. One of the advantages of co-occurrence and correlation network analysis of meta-omics data could be the prediction of the function of the unknown species. Finally, more studies are required to elucidate the role of microbial members of other life domains (e.g., Archaea) in the function and stability of various ecosystems.

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Application of Molecular and Sequencing Techniques in Analysis of Microbial Diversity in Agroecosystem

2

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Abstract

Ecological role of majority of microorganisms including bacteria, archaea and fungi, as well as viruses, is so important that life on Earth would not be possible without them. These microbes interact together with their environment in a very complex way that often defines the community structure and their ecological function. Further due to their extremely small size, there is a huge gap between the present knowledge and predictions. Recent advances in molecular biology especially in the DNA sequencing technology have provided more opportunities for comprehensive studies of these multifaceted microbes in agroecosystems. This chapter focuses on various recent molecular and sequencing techniques used to study microbial diversity.

Keywords

DNA sequencing technology · Microbial diversity · Microbial interactions · Microbial community analysis · Techniques and methods · Agroecosystem

2.1 Introduction

Soil is the important part of the agroecosystems, which consists of huge microbial diversity. In fact soils are highly complex ecosystems of Earth and are the major reservoirs of biodiversity in agroecosystem (Kennedy and Smith 1995; Roger-Estrade et al. 2010; Maron et al. 2011). The soil microbial diversity can directly or indirectly influence crop production, soil health and quality, nutrient uptake and recycling (Roger-Estrade et al. 2010). The soil microbial biota in an ecosystem interact in a relationship such symbiotic, synergism, commensalism, neutralism,

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parasitism, amensalism or antagonism, which may result in a positive or negative impact on the soil function depending on the nature of interaction (Kent and Triplett 2002; Gentry et al. 2015). There are several estimates of the huge soil microbial diversity but are always a matter of debate between the assumptions and the real figures. Reliable assessment of the microbial diversity is of utmost importance for the better understanding of the community structure, function and evolution (Haegeman et al. 2013). The microbes are the regulating factor of ecosystem function; therefore their spatial and temporal distribution is important for the combined understanding (Van Der Heijden et al. 2008). The soil is considered to have 10^8–10^{10} bacteria per gram based on direct counts and 100–10,000 different populations in each gram (Torsvik et al. 1990; Gentry et al. 2015). The commonly known techniques used to assess the diversity are not able to reach the estimated bench mark of over 10,000 distinct organisms per gram of soil (Kent and Triplett 2002). The phylogenetic and functional assessment of the soil microbial diversity is important to understand soil ecological processes. Moreover the microbial biodiversity may also give an idea about richness of other species including plants, animals and microorganisms, while it is relatively a part that includes protozoa, fungi, fauna and bacteria (Chourasiya et al. 2017). The microorganism assemblage is a good indicator of the soil health and productivity (Nielsen and Winding 2002). Further, microorganisms have an important role in nutrient cycling and other ecosystem services. Besides, soil microorganisms function to resistance and resilience of soil-agroecosystem against abiotic stress (Brussaard et al. 2007). Soil microbe is the major factor that checks the invasion of the pathogen in the soil (van Elsas et al. 2012). Microorganisms in the soil are important for the sustainability and nutrient availability of the soil. The soil microbial diversity is a good indicator of soil health (Nielsen and Winding 2002; Anderson 2003; Moura et al. 2018). The microbial diversity can play an important role in the plant residue degradation (Liebich et al. 2007) and helps in the nutrient cycling (Chourasiya et al. 2017). Therefore for the sustainable agroecosystem and for improving the soil fertility and productivity, correct assessment and affecting factors of the microbial communities and diversity are very important. A number of tools are currently being used for the evaluation of microbial diversity, which will be discussed briefly in this chapter.

2.2 Microbial Diversity in Relation to Sustainable Agroecosystem

Ecosystem function is generally directed by means of soil microbial dynamics (Kennedy and Smith 1995). Sustainable agroecosystem is significant to conserve soil properties and qualities while meeting the food production demand. Soil is important part of an ecosystem besides water and air; therefore soil health can influence the sustainability of the ecosystem. Soil health can be described as the ability of soil to provide a vital living system, within the limits of land use and ecosystem, to maintain biological productivity, environmental quality and plant, animal and human health (Doran and Zeiss 2000). Soil holds one of the most complex

biological communities and also much larger portion of the biological diversity in the form of microbial communities. Microorganisms play a key function in soil development, preservation and health. The soil and its microbial diversity have been highly affected due to higher anthropogenic activities (Kennedy and Smith 1995). The soil microbiota perform very important role in ecosystem functioning such as organic matter transformation, biogeochemical cycle and energy flow which considerably influence the sustainability of ecosystem (Choudhary et al. 2018). Further soil microbiota can be affected by the seasonal fluctuations in environmental conditions like temperature, water content and nutrient availability (Torsvik and Øvreås 2002). Therefore, detailed and quantitative investigation of microbial community and structure can be used as an efficient device for assessing soil quality and productivity. The soil microorganisms are engaged in complex interactions in the rhizosphere which affects the plant fitness and soil quality (Barea et al. 2005). The molecular basis plant-microbe interactions in the rhizosphere are still poorly known (Barea 2015). There are several beneficial microorganisms that interact into the rhizospheric zone including non-symbiotic beneficial rhizosphere bacteria and fungi, mutualistic symbiotic N_2-fixing bacteria and arbuscular mycorrhizal fungi (Barea et al. 2005). Soil microbial function is a basic characteristic of sustainable agriculture (Anderson 2003), understanding of which is important for the overall sustainable agroecosystem.

2.3 Advanced Techniques Used for Assessing Soil Microbial Diversity

2.3.1 Fluorescence In Situ Hybridization (FISH)

Fluorescence in situ hybridization has emerged as a significant in situ hybridization technique. It has amenability of integrating with extremely sensitive charge-coupled device (CCD) camera and digital imaging analysis and its efficiency in mapping of numerous probes at the same time (Jiang et al. 1995)**.** Fluorescence in situ hybridization (FISH) is a fairly advanced technique employing fluorescently labelled DNA probe in detection of genes of microbes in soil samples. In fact DeLong et al. (1989) were the first to use fluorescently labelled oligonucleotides for the detection of single microbial cells. It is one of the many nucleic acid techniques that can be used for reliable and rapid identification in their natural habitat. The technique is safer, offers better resolution and has no additional detection steps as compared to radioactive probes. Generally, 15–30 bp oligonucleotide probes, labelled with one or more fluorescent dyes, are used for FISH analysis (Moter and Göbel 2000). Mostly ribosomal RNA (rRNA) are targeted due to their high availability and stability within cells; moreover they possess variable as well as highly conserved sequence domains (Amann and Fuchs 2008). FISH coupled with microautoradiography (FISH-MAR) provides the advantage of in situ identification and offers additional important data on substrate consumption by microbial communities (Zhao et al. 2010). FISH-MAR facilitates the quantitative and phylogenetic characterization of

microbes involved in a process (Rogers et al. 2007). Improved version of the FISH technique is Alexa-FISH that couples with Alexa Fluor dyes, also have better performance and robustness (Mohapatra and La Duc 2012). This technique was successfully used to investigate encapsulation and release of multiple sterilant-resistant *Bacillus pumilus* SAFR-032 spores from poly(methyl methacrylate) (Mohapatra and La Duc 2012). Further the study demonstrated that FISH-based technique can facilitate successful strategies in support of monitoring and mitigating the spore-specific bioburden in a particular milieu (Mohapatra and La Duc 2012). Furthermore FISH technique can also be used for the identification of bacterial colonization (*Folsomia candida*) in the gut and tissue of microarthropods (Thimm and Tebbe 2003). FISH-based techniques have been used to investigate microbial community structure and diversity in soils, sediments, aquifers and other natural habitats (Ainsworth et al. 2006; Rogers et al. 2007). Lenaerts et al. (2007) reported that self-reporting probes such as DNA molecular beacons (DNA MBs) offer better advantages over linear probes for FISH. Further using DNA MBs for FISH facilitated successful detection and cell sorting of spiked and indigenous bacteria from environmental samples like activated sludge and river water, without the need of peptide nucleic acid-based MBs or multilaser flow cytometry (Lenaerts et al. 2007). Extensive autofluorescence of coral tissues and endosymbionts is problematic to use standard FISH method. Ainsworth et al. (2006) demonstrated that combining FISH with spectral imaging can be used to visualize and identify bacterial communities associated with corals. Recently developed web-based tools for FISH allow assistance in probe designing, improving experimental conditions and signal amplification strategies Wagner and Haider 2012). The technique permits culture-independent detection and assessment of microorganisms in their natural habitat. The technique has been used to early monitoring of pathogens such as *Plasmopara obducens* responsible for impatiens downy mildew infection (Salgado-Salazar et al. 2018). In an agroecosystem, early detection of disease causing pathogens and its status can provide useful information to take efficient control measures (Salgado-Salazar et al. 2018). Another application of such technology is in elucidation of host-microbe interaction, that are indeed required for better understanding of the early phases of establishment, later phases lifecycles leading to symbiotic or pathogenic relationship (Remus-Emsermann et al. 2014; Salgado-Salazar et al. 2018).

2.3.2 Community-Level Physiological Profiles (CLPP)

In the late 1980s, community-level physiological profiling (CLPP) owes its first phase to the development of the BIOLOG system. In general BIOLOG is a cultivation-dependent technique that can detect only a part of the entire microbial community (Kirk et al. 2004; Das and Chakrabarti 2013). Initially this system was built in order to detect microbes of clinical value through substrate utilization pattern of 95 unique carbon sources on a single microtitre plate. However, in the 1990s, it was observed that mixed cultures can also be used to get important physiological data of whole microbial community analysed (Silawat et al. 2010). The technique was developed by

Garland and Mills (1991) using a 96-well microtitre plate to evaluate functional diversity of the bacterial community by noting and analysing sole source carbon utilization (SSCU) patterns. Biolog, Inc. (Hayward, CA, USA), commercially provides different types of CLPP plate typically designed for bacteria or fungi (Classen et al. 2003). The GN and GP plates are available to identifying pure cultures of Gram-negative and Gram-positive bacteria, respectively. Both these plates contain 95 different carbon sources and single control well without a substrate. However, some researcher proposed that less than 95 substrates are also sufficient to evaluate changes in functional microbial community in the terrestrial ecosystems (Haack et al. 1995; Lehman et al. 1995). Later ECO plates containing 31 unique C substrates were described by Insam (1997) for bacterial community characterization of environmental samples. To access the fungal CLPPs, SFN2 and SFP2 were developed; these plates have the same substrates as GN and GP plates but without the tetrazolium dye as fungi are unable to reduce (Classen et al. 2003). This method can be used for temporal monitoring of the inoculated populations for their ability to utilize substrates and the speed at which these substrates are utilized (Kirk et al. 2004). Further, the data obtained can be analysed to evaluate the relative differences between functional diversity in soil (Kirk et al. 2004). The method can also be applied to study soil microbes in an edaphically stressful ecosystem. Cartwright et al. (2016) indicated strong correlations between microbial CLPP patterns and different physico-chemical characteristics of soil, essentially soil moisture and temperature differentiating seasonal extremes and thus concluded that soil microbial communities influence various abiotic factors that determine plant community structure of an ecosystem (Cartwright et al. 2016). Frąc et al. (2012) used CLPP to evaluate the effect of organic amendment and mineral fertilization on soil microbial activity and functional diversity. In another recent study, Moscatelli et al. (2018) investigated microbial functional diversity through enzyme activities and CLPP-MicroResp, to show differences among various land use categories. Lagomarsino et al. (2007) recommended that CLPP analysis facilitates the direct assessment of measurement catabolic profile of microbial communities providing an instant outlook of the microbial activity rather than its growth. CLPP is very helpful in identification of copiotrophic microbes from the soil ecosystem as bacterial growth is substrate specific (Lladó and Baldrian 2017). Moreover, CLPP profiles provide useful information about carbon source utilization by soil microorganisms under different cropping systems; the utilization pattern can be helpful for the development of sustainable agroecosystem (Das and Chakrabarti 2013).

2.3.3 ARISA (Automated Ribosomal Intergenic Sequence Analysis)

Automated ribosomal intergenic spacer analysis is a technique for analysing microbial community from different environments and treatments without the prejudice enforced by culture-dependent approaches. Fisher and Triplett were the first who introduced the ARISA and adopted this technique to investigate GenBank database intended for 16S–23S intergenic spacer heterogeneity between cultivated microbes

aimed at detecting effective biases inherent for assessment of microbial diversity offered via this process (Fisher and Triplett 1999). With aim to instant monitoring and assessment of microbial diversity and community structure in natural environment, ARISA was designed to analyse the 16S–23S intergenic spacer region of the rRNA operon with the help of fluorescence-labelled primer. In addition, it can be employed as molecular fingerprinting aimed at assessing variations in community compositions of microorganisms and monitoring of bacterial and fungal communities in fermented food (Fisher and Triplett 1999; Ranjard et al. 2001; Seumahu et al. 2013). Profile patterns of ARISA can be prone to methodological artefacts happening throughout the amplification course. To date, numerous literatures have illustrated the ARISA robustness in generating equivalent profiles in both peak number and intensity as of manifold amplifications as of same and replicate samples. However, ARISA is used in conjugation with 16S-ITS rDNA clone libraries to discover the diversity and composition of marine microbial community, as well as detect assemblages of particular interest which might be particularly targeted for clone library sequencing (Brown et al. 2005). By the application of ARISA, Ranjard et al. (2001) discovered the structure and composition of bacterial and fungal community in various types of soils like LCSA, Montrond, Saint-Elie, OˆMon, Lamto, etc. from various geographical areas with various foliage shelters. Besides, ARISA is also efficient in the investigation of microbial diversity and community structure in freshwater ecosystem in different seasons (Fisher and Triplett 1999), and additionally it may be particularly resourceful at both temporal and spatial scales essential for environmental studies. Furthermore, Fuhrman et al. (2008) used ARISA whole-assemblage genetic fingerprinting in the study of latitudinal diversity gradient in microorganisms such as planktonic marine bacteria, occupied from hemisphere, i.e. tropical to polar. It encourages the notion that metabolism kinetics, setting the stride for life, has great impact on diversity. By using ARISA, examination of OTUs at San Pedro Channel study spot is seasonally varying, annually repeatable and extremely anticipated from environmental parameters, with various OTUs related to different parameters (Fuhrman et al. 2008; Fuhrman et al. 2006). It is a suitable and appropriate tool in diverse environments comparison and various treatments, along with the benefit of still being substantially not as much of expensive in comparison of pyrosequencing. In addition, it may serve as a good cross-validation process for pyrosequencing techniques (Jami et al. 2014). ARISA also have application in the assessment of microbial dynamics in cheddar cheese in salt level and type, and it can be employed as fingerprinting technique, verified to be a fast and low-cost practice intended for the discrimination of lactic acid bacteria (LAB) in cheese and illustrated better resolution and performance than DGGE (Porcellato et al. 2014). In a study conducted by Abed et al. (2014) using ARISA technique on microbial diversity, distribution and potential of hydrocarbon biodegradation in oil contaminated by cyanobacterial mats from a constructed wetland revealed that various mats comprised diverse microbial communities. Moreover, ARISA have also application in the study of microbial diversity as well as microbial community composition in grape juice at wine fermentation (Ghosh et al. 2015). B-ARISA is also a potential device in identifying bacterial

communities particularly for simple communities arising in a limited zone or a controlled system with recognized bacterial community composition and biases (Purahong et al. 2015).

2.3.4 DNA Barcoding

Barcoding defined as DNA-based species identification at molecular level and transformed the traditional method to the study of biodiversity sciences (Cristescu 2014). DNA barcoding technology is a cost-effective technology, permits delicate and multiplexed target analysis (Agasti et al. 2012) and has potentials of rapid, precise identifications of microorganism with concentrating examination on minute standardized section of genome (Hebert et al. 2003). DNA barcode markers used to particular taxonomic groups of microorganisms are indispensable for understanding species boundaries, functional trait evolution, community ecology, the conservation of biodiversity and trophic interactions (Kress et al. 2015). By means of identification tools, DNA barcoding is based on the generation of great quality of reference database of sequence that has been utilized to develop phylogenetic trees intended for employ in ecology of phylogenetic community (de Vere et al. 2012). Arnot et al. (1993) introduced the concept of DNA-based identification of species, and further Hebert et al. (2003) standardized this concept. Nowadays, DNA barcodes are utilized in identification of specimens, environmental management, conservation of biodiversity, trophic-level association study, food safety and invasion biology (Cristescu 2014). According to Ji et al. (2013), metabarcoding approach encompasses DNA-based identification of species to individual's community. Closeness to several group of species has diverse functions in ecosystem, and approach like DNA barcoding employs vast parallel sequencing of whole samples, i.e. total DNA or degraded DNA obtained from environmental samples (eDNA) for which species identification with accuracy is not practical (Cristescu 2014). Kamo et al. (2018) developed DNA barcoding methods for identification and quantification of the species of European honeybee from each pollen pellet. de Vere et al. (2012) present DNA barcode resource for 1143 species that include native flowering planta and conifers for nation of Wales. In animal group, DNA barcode have been applied in restricted number with the application of cytochrome C oxidase subunit 1 and mitochondrial gene (Cowan et al. 2006). As stated by Savolainen et al. (2005), DNA barcodes comprise of a standardized short DNA sequence between 400 and 800 bp that might comfortably be produced and characterized for each species present on the Earth. Several studies have been conducted through DNA barcoding to classify *Mentha* species phylogenetically, correct identification and fix the problem of adulteration of the medicinal plants that belong to Lamiaceae family, in species-level identification of duckweeds, in identification of phytoplankton of Persian Gulf, in identification of animal species in traditional medicine and in identification of flowering plants (Thakur et al. 2016; Zahra et al. 2016; Wang et al. 2010; Alemzadeh et al. 2014; Yang et al. 2018a; Kress et al. 2005). Metabarcoding can be defined as potential approach that is interpreting the additional information that make the

traditional DNA barcoding irrelevant (Taylor and Harris 2012). It has been reported that numerous studies of metabarcoding performed on microbial communities have substantial rare species diversity in comparison to classical morphology (Sogin et al. 2006).

2.3.5 RFLP (Restriction Fragment Length Polymorphism)/ ARDRA (Amplified Ribosomal DNA Restriction Analysis)

Over the last two decades, restriction fragment length polymorphism (RFLP) or amplified ribosomal DNA restriction analysis (ARDRA) has been widely used to study microbial diversity. The technique is based on DNA polymorphisms (Kirk et al. 2004). PCR-amplified rDNA is digested with a four-base-pair cutting restriction enzyme, followed by the detection of the different fragment lengths through electrophoresis. The method gives very less or no information regarding the type of microbe, but it can be useful for quick monitoring and comparison of microbial communities over time and/or in response to variable conditions (Agrawal et al. 2015). The method can be used for the microbial community fingerprinting (Massol-Deya et al. 1995). ARDRA profile has been used to manually group similar isolates especially when the number of isolates is quiet large for further handling (Hall et al. 2001; Lima-Bittencourt et al. 2007; Dhal et al. 2011; Croes et al. 2015; Mukherjee et al. 2016). ARDRA can be used in grouping at the species and genus level, for example, *Lactococcus*-like strains into *Lactococcus* and *Enterococcus* species and differentiation of *L. lactis* strains at subspecies level (Delgado and Mayo 2004). The method is also used for identifying the unique clones and estimating OTUs in environmental clone libraries (Smit et al. 1997; Dhal et al. 2011). The method has been used to study microbial diversity in wastewater (Smit et al. 1997; Chandra et al. 2010), soil (Aquilanti et al. 2004), root nodules (Ibáñez et al. 2009), radioactive contaminated wastes (Dhal et al. 2011) and rumen (Natsir et al. 2016).

2.3.6 T-RFLP (Terminal Restriction Fragment Length Polymorphism)

Terminal restriction fragment length polymorphism (T-RFLP) is the extended and improved technique with simpler banding patterns than RFLP (Kirk et al. 2004). Initially the method was developed for rapid identification of mycobacteria (Avaniss-Aghajani et al. 1996). It follows a similar principle as RFLP, but one end of PCR amplicons is tagged with a fluorescent molecule (e.g. TET (4,7,2V,7V-tetrachloro-6-carboxyfluorescein) or 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein)) attached to a primer (Blackwood et al. 2003). The amplified product is then ligated using a restriction enzyme, and the generated terminal restriction fragments are subsequently separated by electrophoresis and visualized by excitation of the Fluor (Blackwood et al. 2003). The generated banding pattern is used to measure species richness and evenness as well as similarities or

dissimilarities between various environmental samples (Liu et al. 1997). T-RFLP profiles are relatively stable to variability in PCR conditions (Osborn et al. 2000; Ramakrishnan et al. 2000). Blackwood et al. (2003) described T-RFLP as a sensitive and rapid tool that can be applied for the quantitative comparison of microbial communities. The technique do has some limitations such as incomplete digestion by restriction enzymes and low availability of template DNA of the rare species that may lead to inaccurate estimation (Liu et al. 1997; Osborn et al. 2000; Kirk et al. 2004). The DNA banding profiles can also vary with the enzyme used (Dunbar et al. 2000). Further Dunbar et al. (2000) combined the profile data of different enzymes and observed no significance between different samples. Osborne et al. (2006) reported that using multiple restriction enzymes to generate multiple data sets enables confident estimate for groupings of apparently similar communities and lesser effects of enzyme selection. However, despite the limitation, general view is that the technique can be standardized to measure spatial and temporal differences (Liu et al. 1997; Osborn et al. 2000). De Vrieze et al. (2018) suggested that T-RFLP is a reliable technique for swift microbial community screening. T-RFLP profiles generated by an automated electrophoresis system can be analysed quantitatively using either peak height or peak area data, but peak height data showed better reproducibility than peak area data (Caffaro-Filho et al. 2007). T-RFLP has been effectively used for the characterization and differentiation of microbial communities in various environments such as archaeal populations in the marine fishes (Van Der Maarel et al. 1998), archaeal community in anoxic rice field soil (Ramakrishnan et al. 2000), microbial diversity in different soils (Dunbar et al. 2000), microbial communities from different agroecosystems (Blackwood et al. 2003), arbuscular mycorrhizal fungal communities and composition in grassland microcosms (Johnson et al. 2004), ectomycorrhizal community associated with 125-year-old *Pinus sylvestris* L. (Genney et al. 2006), microbial community structure of activated sludge (Hoshino et al. 2006), endophytic bacteria associated with *Tricholoma matsutake* fruiting bodies (Li et al. 2016), microbial communities in groundwater ecosystems (Karczewski et al. 2017) and monitoring of plant growth-promoting rhizobacteria in rhizosphere of *Zea mays* L. (Kari et al. 2019).

2.3.7 RAPD (Random Amplified Polymorphic DNA)

RAPD (random amplified polymorphic DNA) is described as PCR-dependent technique for detecting genetic divergence; however, amplified DNA segments are random. It is relied on principle that a single, short oligonucleotide primer associated with several diverse loci is applied for amplification of random sequences by complex DNA template, and it suggests that the amplified segment produced through PCR based on the length and size of both the primer and the target genome (Kumar and Gurusubramanian 2011). In comparison to isolate- based methods for assay of community structure, RAPD is simple, quick and more meaningful, which makes it one of the popular DNA-based techniques for assessing soil microbial community structure (Xia et al. 1995; Yang et al. 2000). Its another application developed

measurable approximation of the comparative amount of several genomes in mixed DNA samples (Hadrys et al. 1992). A study conducted by Brandt et al. (1998) on *Aspergillus fumigatus* for analysis of taxonomy through random amplified polymorphic DNA (RAPD) PCR (RAPD-PCR) cloning and the TaqMan LS50B fluorogenic detection system. In addition, such analysis was utilized in screening of *Aspergillus fumigatus* DNA designed for species-specific amplicons. Harry et al. (2001) have successfully applied RAPD technique to compare the three tropical soil microbial communities. Franklin et al. (1999) used RAPD to evaluate the microbial community of two aquatic systems. In a study on microbial community diversity from four soil samples that were affected by agriculture chemicals, Yang et al. (2000) used 14 random primers that generate 155 reliable fragments as products of 12 primers, of which 134 were polymorphic. This study clearly indicates the diversity of microbial community in soil is affected by chemicals used in agriculture. There is certain limitation of RAPD technique; it is impossible to detect if changes take place below 1% in total DNA content (Xia et al. 1995).

2.3.8 DGGE and TGGE (Denaturing and Temperature Gradient Gel Electrophoresis)

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are PCR-based methods. DNA is extracted from natural samples, and a part of the 16S or 18S rRNA sequences are amplified using PCR using universal primers. The amplified sequence of the same length but with different base-pair sequences is separated based on the difference in mobility of partially melted DNA sequences in acrylamide gels containing a linear gradient of DNA denaturants (urea and formamide) or temperature gradient. Originally these methods were developed to identify point mutations in DNA sequences (Kirk et al. 2004) and were later described to evaluate the microbial genetic diversity (Muyzer et al. 1993). Thus these methods are principally based on the separation of ribosomal gene sequences amplified directly from community DNA employing conserved primers on a denaturing gel based on melting behaviour of the double-stranded DNA (Riesner et al. 1989; Eichner et al. 1999; Muyzer 1999). Therefore these methods essentially do not require microbial culture in the laboratory to study total microbial population. Øvreås and Torsvik (1998) compared the culturable bacterial diversity with total bacterial population in organic soil and sandy soil through ARDRA and DGGE analysis. Further they observed that dissimilarity in the bacterial diversity between the two soils was largely due to the fraction of the uncultivable bacteria; thus extraction and analysis of total DNA from microbial populations are significant for the overall functionality (Øvreås and Torsvik 1998). After the first publication by Muyzer et al. (1993), an increasing number of studies have employed DGGE/TGGE to access the microbial communities and ecology (Muyzer 1999; Gómez-Villalba et al. 2006; Nakatsu 2007; Wang et al. 2008; Sun et al. 2013). Theoretically, the method separates DNA sequences having a difference in only one base pair (Miller et al. 1999). The methods are reliable, reproducible, fast and

comparatively inexpensive (Kirk et al. 2004). In addition multiple samples can be studied simultaneously; therefore variation in several microbial communities against any stimuli/adversity can also be detected (Muyzer 1999). DDGE allows easy separation of PCR amplicon of the nucleic acids directly extracted from soils samples without the need of culture and purification of microbe in the laboratory (Nakatsu 2007). Therefore numerous samples can be analysed and compared simultaneously in relatively less time. However, there are certain limitations that could potentially influence the microbial community, including laborious sample handling, DNA sample extraction error and PCR amplification-based errors, co-migration, gel constraints, GC clamp disadvantage and presence of heterogenous genes in samples (Wintzingerode et al. 1997; Muyzer 1999; Theron and Cloete 2000; Adil 2015). TGGE analysis based on partial sequence of the 16S rRNA gene of bacterial community composition of biofilms formed on a submerged column of biofilter system was found to be stable during study period of more than 1 year (Gómez-Villalba et al. 2006). Sun et al. (2013) coupled DGGE analysis with adenosine-triphosphate analysis and testing of mixed liquid and suspended solids to alternation in microbial activity and community structure during treatment process of biological wastewater of tomato paste. The study established that the microbial community structure and quantity were directly related to mixed liquid and suspended solids during the wastewater treatment process. Zhang et al. (2005) recommended that sequence heterogeneity in single denaturing gel electrophoresis (DGE) bands could be overestimated due to single-stranded DNA (ssDNA) contamination. Further they suggested to minimize ssDNA contamination through purification, before constructing a clone library to retrieve the sequence diversity of a single DGE band. Wang et al. (2008) used DGGE to investigate the changes in the bacterial populations during microbial enhanced oil recovery. DGGE/TGGE techniques have also been used to assess communities of actinomycetes in various samples (Heuer et al. 1997; Bora 2015). Kadali et al. (2015) demonstrated RNA TGGE can be used as effective and reproducible technique to investigate microbial communities during commercial bioremediation process in a marine ecosystem for the degradation of hydrocarbons. In recent study, PCR-DGGE was used to determine effect of biostimulation treatment using composted plant biomass on bacterial diversity of petroleum-contaminated soil (Solomon et al. 2018). In another study, changes in soil bacterial diversity of the antibiotic-contaminated soil and/or treated with a multidrug-resistant *Raoultella* sp. strain were investigated using PCR-DGGE approach; further, it was observed that antibiotic application into soil may cause temporary risk to soil functioning (Orlewska et al. 2018).

2.3.9 DNA Microarray Technology

Since 1995, DNA microarrays were generated for profiling of gene expression, and it consists of 100 or 1000 of DNA fragments arrayed on small glass slides (Schena et al. 1995). This technology is a potential tool for quick monitoring and assessment of microbial diversity and functions. An individual array can hold thousands of DNA

sequences which enables broad hybridization with large-scale identification potential (Cho and Tiedje 2001). Kjelleberg (2002) introduced the term 'environmental microarray' for environment-related studies in microarray. Various types of probe such as oligonucleotides, cDNA and microbial genomes have been used by DNA microarrays intended for employ in microbial ecology such as environmental microarrays, microbial diagnostic microarray or microbial ecological microarrays (Roh et al. 2010). Universal DNA microarray has potential to analyse cyanobacterial microbial diversity (Castiglioni et al. 2004). Microarray may consist particular target genes, for instance, nitrate reductase, nitrogenase or naphthalene dioxygenase to deliver functional diversity data, or may consist 'standard' of environmental samples (DNA fragments with >70% hybridization) showing diverse species that originate in the environmental sample (Cho and Tiedje 2001). DNA microarray used in the investigation to quantify the functional gene diversity in nitrogen cycle in the surroundings, such technologies, has various applications in environment, for example, analysis of gene expression profiling, assessment and genotyping of microorganism dependent upon genomic DNA-DNA resemblances, population genetics and functional genes discovery (Taroncher-Oldenburg et al. 2003). Besides, this technology also has role in microbial ecological studies, for instance, assessing and estimating several gene families engaged in nutrient cycling, biodegradation and pathogenesis (Tiedje et al. 2001). Microarray analysis is emerging a widespread technique in several fields of microbial research comprising microbial physiology, epidemiology, phylogeny, ecology, pathogenesis pathway engineering and fermentation optimization (Rick et al. 2001).

2.3.10 Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) is currently a promising and cost-effective tool for quantitative analysis of microbial communities in various environmental samples (Fierer et al. 2005; Novinscak and Filion 2011; Sirohi et al. 2012; Demidenko and Penin 2012). The method can be applied to investigate gene expression profiling of microorganism in soil (Piveteau et al. 2011; Demidenko and Penin 2012). The technique is based on the real-time monitoring of a reporter molecule whose fluorescence increases as the amplicon increases during reaction cycle (Raeymaekers 2000; Fierer et al. 2005; Derveaux et al. 2010). There are two major fluorescent reporter molecules that generate fluorescence: (i) SYBR Green (or other intercalating dyes) and (ii) hydrolysis (TaqMan®-style probes). The concept of the qRT-PCR was given by Higuchi et al. (1992). Later the concept was developed to first real-time PCR machines (Wittwer et al. 1997a, b). Since then the technique has been widely used in genomics, medical science, agriculture and environmental studies (Wong and Medrano 2005; Fierer et al. 2005; Zhang and Fang 2006; Valones et al. 2009; Beaulieu et al. 2011; Doi et al. 2015; Kredics et al. 2018; Yang et al. 2018b). The technique is rapid and allows accurate detection of the changes in gene expression as a result of physiology, pathophysiology or developmental variations (Valasek and Repa 2005). The method can be used to assess responses to

experimental stimuli and changes in protein level and function (Valasek and Repa 2005). Although PCR-dependent methods can be used to detect DNA or RNA at low amounts, accuracy is dependent on number of factors; however, qRT-PCR can be used for the estimation of microbes at low concentration levels (Zhang and Fang 2006). Fierer et al. (2005) described qRT-PCR as an important tool to characterize soil microbial communities. They further examined soil bacterial and fungal community structure by qRT-PCR to revel the abundance of the respective taxonomic groups (Fierer et al. 2005). Antonella and Luca (2013) reported that qRT-PCR can be an efficient molecular tool for the study of phytoplankton cells at the pre-bloom levels; further the methods can also be applied to the preserved environmental samples. The analysis of the qRT-PCR data is important part of any experiment, but some of the available tools provide only a particular part of analysis, while others cover complete analysis (Pabinger et al. 2014). Offered functionality, features (graphical format of the data presentation and statistical methods) and specific requirements (data format) of the software and experiment design should be considered while selecting any tool for the analysis. Further Pabinger et al. (2014) suggested that standardized format should be adopted to make it easy to transfer data between instrument software, analysis tools and researchers (Pabinger et al. 2014).

Reverse transcription with subsequent PCR can be applied to measure mRNA with high sensitivity (Saleh-Lakha et al. 2005). The method uses total isolated RNA to reverse transcriptase to make cDNA copies that are subsequently used as templates in a PCR reaction along with probes designed to amplify the genes of interest (Saleh-Lakha et al. 2005). The real-time quantitative reverse transcription-PCR (RT-qRT-PCR) can be used for the quantification of microbial gene transcripts. Gao et al. (2011) reported that this technique is highly sensitive enough to measure gene expression of a single bacterial cell and the gene expression heterogeneity among the bacterial cells. The method is best suited for the detection and quantification of gene expression in environmental and soil samples (Sharkey et al. 2004; Saleh-Lakha et al. 2005). DeCoste et al. (2011) monitored quantification of *hcnC* and *phlD* gene transcripts using qRT-PCR in natural soil amended with *Pseudomonas* sp. strain LBUM300.

2.3.11 Pyrosequencing

The current molecular microbial ecology tools, such as TRFLP, DGGE or TGGE, facilitated culture-independent identification and diversity analysis of microbial populations in various environments, but the overall diversity estimates of these methods are usually low as compared to the predicted values (Li et al. 2014). These restrictions can be overcome by using newly developed DNA sequencing approaches such as pyrosequencing. Pyrosequencing is next-generation sequencing technology that involves synthesis of complementary strand one base at a time and at the same time determines specific nucleotide being incorporated in the synthesis process (Cummings et al. 2013).

Wang et al. (2012) observed 60 unique bacterial genera by using high-throughput pyrosequencing in the activated sludge samples from 14 wastewater treatment plants located across 4 different cities in China. Daquiado et al. (2016) determined the structure and diversity of bacterial communities in a long-term fertilized paddy field soil ecosystem and further evaluated the differential effects of organic and inorganic fertilizer application to the soil. The study successfully established that organic fertilizers can be applied without any negative effects and compromising soil microbial diversity in the long-term use (Daquiado et al. 2016). Jackson et al. (2013) compared the plant-associated microorganisms of the commercially available salad leaf vegetables by the culture-dependent and culture-independent pyrosequencing; further low-abundance bacteria that were not detected by culture-dependent methods were identified by pyrosequencing. Although pyrosequencing is relatively more expensive but provides an efficient method to evaluate microbial diversity, assuring high bacterial richness coverage as compared to culturable method and DGGE (Vaz-Moreira et al. 2011). In a microbiome study of the intensive care units of hospitals, more diverse sequences were detected through pyrosequencing as compared to traditional culture and characterization (ARDRA) method that only detected 2.5% of the total bacterial diversity (Oberauner et al. 2013). But the fact that pyrosequencing-based sequencing could detect DNA from nonliving and living microbe should also be taken into account (Oberauner et al. 2013). Pyrosequencing has been extensively employed to carry out metagenomic studies on microbial communities from various environments such as microbial diversity of soil (Terrat et al. 2012), microbial community of an acidic hot spring (Bohorquez et al. 2012), rhizospheric communities of mangroves (Alzubaidy et al. 2016), microbial communities from hydrocarbon-contaminated soils (Stefani et al. 2015), total bacterial communities of *Cucurbita pepo* ssp. *pepo* grown under the influence of organic pollutants and without it (Eevers et al. 2016), fruit-associated fungal communities (Taylor et al. 2014), endophytic bacterial communities over three successive generations of *Crotalaria pumila* from metal contaminates sites of mining area (Sánchez-López et al. 2018), microbial communities of browning Peninsula of eastern Antarctica (Pudasaini et al. 2017) and soil microbial diversity of the site under remediation with *Miscanthus x giganteus* (Bourgeois et al. 2015).

2.3.12 Illumina Next-Generation Sequencing

In the recent times, Illumina-based sequencing has emerged as potential tool to study microbial diversity. Illumina-based sequencing is less error prone and is capable of providing higher phylogenetic resolution as compared to 454 sequencing (Shi et al. 2014). Additionally, Illumina can provide 30 times more reads than 454 sequencing that provides a platform to perform in-depth sequencing of samples in a single run (Wang et al. 2016). The first commercially introduced Illumina (Solexa) platform produced reads with 35 bp length with a focus on genome sequencing (Van Dijk et al. 2014). But later the technology improved, and read lengths were achieved by merging paired-end reads, thus making it suitable for the analysis of environmental samples (Tan et al. 2015). Illumina-based sequencing

can now generate reads of several hundreds of bp long (Van Dijk et al. 2014). Illumina-based sequencing is basically a 'sequencing-by-synthesis' method that uses fluorescently labelled nucleotide base called reversible terminators. Illumina-based sequencing is an excellent technique to study plant-associated microbes in the agriculture systems (Wang et al. 2016). Siddique and Unterseher (2016) described Illumina-based sequencing as a cost-effective and efficient method to study microbial community composition in different physiological states. Sharma et al. (2019) evaluated endophytic community in a hyperaccumulator plant growing naturally in the soils contaminated with potentially toxic metals through Illumina sequencing. The Illumina-based sequencing has been widely adopted to investigate the microbial diversity in various environments such as endophytic bacterial diversity of sugar beet (Shi et al. 2014), fungal diversity of calcareous deep-sea sediments (Zhang et al. 2016), endophytic microbiota in *Oryza sativa* L. (Wang et al. 2016), fungal diversity in dates (Al-Bulushi et al. 2017), soil microbial community of ginger cultivation field (Liu et al. 2017), microbial community and succession during 60 days of sludge composting (Wang et al. 2018) and fungal communities in the Arctic marine sediments (Kabeer et al. 2019). The Illumina platform is capable of producing soil metagenomes up to 0.4–29 million reads per sample with a maximum metagenome size of about 4.0 Gbp (Myrold et al. 2014). The soil metagenome generated through Illumina can provide huge information that is helpful in better understanding of taxonomic and functional diversity of soil microorganisms (Myrold et al. 2014).

2.4 Concluding Remarks

Molecular methods have the potential to provide vital knowledge about the microbial diversity in soil agroecosystem. The knowledge can be applied for the management of microbial interactions and the manipulation of suitable microorganism for the sustainable agriculture practices and food security. Further, the continuous advancement in the modern molecular tools guarantees much accurate and faster characterization and assessment of microbial diversity in the future.

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3 Bioinformatics Resources for Microbial Research in Biological Systems

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Abstract

Bioinformatics is a continuously evolving field since it came into existence and contributing significantly in all major areas of biological sciences. Advanced sequencing technologies and exponential growth in computational resources have facilitated the high-end bioinformatics application in various research areas such as microbiome research in biological system. Bioinformatics contributed significantly in the development of powerful methods and tools in metagenomics research through direct inspection of targeted and nontargeted DNA in environmental samples. Advances in metagenomics, high-throughput methods, tools, software, pipelines, databases and analysis products for the microbes and microbiome-related studies have shifted the field of microbiology from culturing and microscopy studies to DNA sequencing and bioinformatics analyses. In the last decade, various long-term research projects and studies have flooded the microbiome sequencing data and analyses. Now, microbial community is realized that the next decade of microbial research will need data management, sharing, mining and networking skills to enhance knowledge discovery and regulation of microbial communities in ecosystem. Here, we are describing the microbiome researches in different biological domains, microbial databases and tools, which can be useful for application of microbes in emerging applied fields.

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Metagenomics · Bioinformatics · Microbes · Plant-microbe interaction · Human-microbe interaction

3.1 Introduction

Bioinformatics was originated as technique for the information processing in biological data.

However, later, bioinformatics has evolved and become an interdisciplinary research field. As discipline, bioinformatics has broad aims: (i) organize data in a way that allows scientists and researchers to produce new knowledge and (ii) develop algorithms, tools and resources for easy data analyses and interpretation in meaningful manner (Luscombe et al. 2001). In the past four decades, bioinformatics has extended its potential in various complex studies, method developments and knowledge discoveries and established as "Research Driving Discipline" with the advancement in sequencing and computational technologies (Azuaje et al. 2012).

The role of microbial communities in biological system was well known since the beginning of microbiology research. But structural and functional understanding of microbial communities was less known before 1975, due to available techniques and methods. In the 1990s, microbiology research started to transform with the advancement of molecular techniques, sequencing methods and computational infrastructure, and metagenomics evolved as a new research area for microbiology (Hugenholtz 2002; Hiraoka et al. 2016). The major obstacle in microbial research was uncultivable environmental microbes. The community of microbial scientists solved this hurdle through the development of culture-independent methods such as microarray, PCR and DNA cloning techniques which have been used to detect specific species and functional genes in microbial communities (Su et al. 2012). Large-scale sequencing problem of environmental DNA was resolved through modern sequencing technologies and methods such as shotgun metagenomics and (16S, 18S and ITS) amplicon sequencing, which enable us to identify individual microbes and their functional genes at large scale with more detail. These kind of techniques have been used in diverse environment to reveal the presence of microbes in soil (Manoharan et al. 2017), plant-microbe interaction (Schirawski and Perlin 2017) and microbes and human relation and also characterized the nutrition systems involved in symbiosis (Erickson et al. 2012; Woyke et al. 2006). Now, various large-scale metagenomic projects are generating comprehensive microbial sequence data for diverse environments such as plant- and human-associated, soil, fresh water and ocean environments.

The advances in this field has increased the bioinformatics outreach in microbiome analysis and informatics and is expected to accelerate the microbial research. A large number of bioinformatics tools, software and pipelines were developed and very useful to analyse metagenomic sequence data such as MEGAN, QIIME, Mothur and MG-RAST, which allow researchers to perform integrated analyses and

visualize at large scale (Oulas et al. 2015). The rapid growth of metagenomic data and bioinformatics resources will identify potential field for discoveries and application and hypothesis-driven and targeted data generation in the future.

3.2 Plant-Microbe Interaction: A Key for Sustainable Agriculture Production

The agriculture productivity highly depends on the physical and biological quality of soil. Enhancement of biological quality for beneficial plant-microbe interaction could be one of the approaches for sustainable crop yield in the future. The current progresses in computational resources and high-throughput techniques (DNA microarrays, next-generation sequencing, proteomics and metabolomics) for the processing of a large number of biological samples have increased the pace of systems biology research in plant science (Yadav et al. 2017). It is speculated that ten billion microorganisms and thousands of different species are present in just 1 g of soil (Knietsch et al. 2003). In the field, plants are likely exposed to a high number of microbes, but we do not fully understand how many of these are beneficial, helping in resistance, or act as biocontrol agent. The ecological communities of plant-microbe are exaggerated by plant genotype and different ecological factors. Plant-microbe interaction drives plants to release different kinds of metabolites such as carbohydrates, amino acids, organic acids, nucleotides, glucosinolate, flavonoids, antimicrobial compounds and enzymes which makes a difference between plant genotypes for physiological and immune responses and leads to create host-specific microbial communities (Musilova et al. 2016). Although metagenomic sequencing reveals microbial identities and functional gene information at large scale, the combined understanding of genetic potential of the microbiome and available soil resources is yet to be explored for sustainable agricultural practices (Nesme et al. 2016). Some of the important projects have been initiated to explore the soil taxonomic and functional diversity of microbes at large scale for the purpose such as the Earth Microbiome Project, TerraGenome, the Brazilian Microbiome Project, the China Soil Microbiome Initiative, etc.

3.2.1 Plant-Microbe Interaction

In nature, plants and microbes are highly dependent on each other for development, defence and adaptation against environmental shift. Once this mutualism is disrupted, the plant-microbe interaction becomes very sensitive and demands human intervention for defence and environmental shift (Bulgarelli et al. 2015; Levy et al. 2018). Genetic information encoded in plant genome has potential to affect the structure of their microbiome in favour of beneficial microbes as well as against pathogens. Similarly, microbes have also potential to alter the host plant for their own benefit, e.g. altering host metabolism for metabolites. The majority of plant-associated microbial communities are found in the rhizosphere and phyllosphere of

plants, where they lie on plant tissues or on the surface of plant and produced beneficial and harmful activities for the plant. Moreover, function of a large number of microbes in plant communities is not clear yet for their contribution (Levy et al. 2018). Network analyses of plant-related communities have shown the importance of strongly interconnected microbial taxa as “microbial hubs”, as a key to understand microbiome dynamics, and the effect of single microbes on the structure of microbial communities (Agler et al. 2016). It also suggested that small changes could lead to significant effects on the composition of plant microbiome. In recent year’s studies, application of microbiota for new plant varieties has been witnessed. It was found that some microbes provide services to the plant that used to replace traits lost during breeding (Mundt 2014). Interestingly, it is also found in a study that leaf metabolite and insect feeding behaviour can also influence the rhizosphere microbiome composition and prolonged insect feeding can also reshape the overall root microbiome structure (Badri et al. 2013). Influence of soil type and plant cultivar effect on the structure of rhizosphere were studied for *Verticillium dahliae* (Nallanchakravarthula et al. 2014), and specific suppressiveness potential of soil was also studied against *Fusarium* wilt in strawberry (Cha et al. 2016). In brief, these studies have demonstrated the potential of plant-microbe interactions to produce significant effect on plant phenotypes without changing plant genomic information.

Recent development in metagenomics techniques has opened the opportunities to overcome the soil ecosystem complexity and build our fundamental knowledge in the area of plant-microbe interactions. In current, microbial genomics is equipped with a series of powerful techniques to differentiate beneficial, neutral and harmful microorganisms at community and individual microorganism level with next-generation sequencing (NGS) platforms such as 16S/18S/ITS amplicon sequencing, metagenomics, metatranscriptomics, complete plasmid sequencing and microbial single-cell sequencing (Jansson and Hofmockel, 2018; Nesme et al. 2016). Application of plant-microbiome interaction for agriculture can be improved through the understanding of soil microbial diversity, functional microbial genomics, biological products and bacterial genome modification with the help of current high-resolution and high-throughput molecular techniques.

3.2.2 Plant Microbial Diversity Analysis

Plant-associated microbial communities have a significant role in regulating nutrient cycling, carbon mineralization and stabilization. It is also found in various studies that belowground microbial communities have high influence on aboveground biotic communities such as plant species diversity and productivity. So, abundance and diversity of plant-associated microbial communities are biological indicators of crop health and support a balanced and sustainable ecosystem. In recent years, the significance of microbial influence for plant health and fitness has been stressed, and it has been suggested that microbial diversity in the plant microbiome may act as an outer layer of plant immunity (Berendsen et al. 2012). It is very difficult to

isolate vast majority of microorganism through traditional and culture-dependent methods to study microorganisms in their natural environment. Metagenomic studies directly from the environmental genetic material have benefitted significantly for the exploration of microbial biodiversity through NGS technologies.

To the study of microbial diversity, 16S/18S/ITS sequencing is a powerful and most commonly used method. In 16S/18S/ITS amplicon sequencing, sequencing platforms such as Illumina, Oxford Nanopore or PacBio are used to read the amplified PCR products with suitable universal primers of one or several regions of 16S/18S/ITS. 16S and 18S rDNA are hypervariable regions in the 16S or 18S rRNA genes in bacteria and fungi, while ITS (internal transcribed spacer) is the spacer DNA between the small and large subunit of rRNA genes in bacteria, fungi and archaea. In bacterial diversity studies, 16S and 18S rDNA marker sequences are used. These marker sequences consist of nine variable regions (V6 absent in 18S) and ten conserved regions; the conserved region sequences reflect the genetic relationships between species, and variable region sequences indicate the difference between species. ITS sequence markers, part of the non-transcriptional region of the fungal rRNA gene, are used for fungal microbial community analysis. 5.8S, 18S and 28S rRNA genes are highly conserved in fungi. However, ITS region allowed tolerating more mutations in the evolutionary process due to low natural selection pressure and exhibits wide sequence polymorphism in eukaryotes (Martijn et al. 2017; Ni et al. 2008). After generating various isoforms of targeted genes through amplicon sequencing, taxonomic composition of microbial communities can be determined, and the probable relation of high-disease field by comparing microbiomes between high-disease and low-disease field or difference between high-yield and low-yield plants can be understood. Artificial manipulation of microbial diversity may increase crop yield, such as addition of beneficial microbial strains as biocontrol agent for the restriction of pathogenic microorganism.

3.2.3 Microbial Products in Agriculture

Identification and characterization of microbial community for disease control, cope with stress, plant growth and development is the most important aspects of microbes lead agriculture industry. Numerous kinds of microbes especially bacteria, i.e. *Rhizobium*, *Bacillus*, *Pseudomonas*, *Pantoea*, *Paenibacillus*, *Burkholderia*, *Achromobacter*, *Azospirillum*, *Microbacterium*, *Methylobacterium*, *Variovorax* and *Enterobacter*, are known to help plant growth and development. These mentioned microbes known as plant growth-promoting bacteria (PGPB), by releasing the biochemical compounds to regulate controlling essential hormone, provide nutritional support in abiotic stresses like drought, excessive water, heat, osmotic and salt (Grover et al. 2011). Microbial biofertilizers are used to fix atmospheric nitrogen and make it available as $NH4+$ for plants by associating symbiotically with root or other plant organs. Nevertheless, some microorganisms also perform nitrogen fixation in free-living condition (Burr et al. 1984). A compendium of microbes have been used as bioproducts for various biological controls of pests and diseases, for

example, *Pseudomonas* and *Bacillus subtilis* species are used against *Botrytis* and *Fusarium* spp. control. In 1895, Nitragin was the first patent for *Rhizobium* species based product on nitrogen-fixing bacteria. Today, a large number of nitrogen-fixing bacteria are produced industrially and marketed with high commercial impact in the USA, Canada, Australia, South Africa, Israel and other countries of Asia. However, the quality and efficiency of inoculants are not consistent in changing climatic condition, which is a big obstacle for microorganisms, thus enabling product development (Martínez 2015). The recent advanced technologies, such as metatranscriptomics, and single-cell sequencing techniques can help scientists to develop microbial products with durable efficiency to increase crop yield by optimizing beneficial bacteria or protecting plants from pests and disease.

3.2.4 Microbial Genetic Engineering for Production

Genetic engineering is used to produce ethanol, vitamins, enzymes, carbohydrates and chemical precursors from bacteria such as *Bacillus subtilis* and *Escherichia coli* (Reuß et al. 2017). Fungus *Ashbya gossypii* is used for the production of riboflavin production (vitamin B2), which increased the production efficiency and reduced the environmental protection costs by 43% and 30%, respectively, in comparison to chemical synthesis (Wenda et al. 2011; Iniesta et al. 2016). Genetically modified organisms can play a major role in meeting our future demand for refined industrial products and to fight major threats to ecosystems. Identification and construction of desired strains is one of the major challenges for enabling microorganism production due to high mutation rate and changing climatic conditions. Microbial genetic engineering has evolved and is continuously evolving to handle challenges and associated complexities such as targeted evolution studies, native pathway modification, non-native pathway and synthetic pathway introduction and introduction of natural and synthetic pathways into synthetic organisms (Portnoy et al. 2011; Dragosits and Mattanovich, 2013). At the present time, it is possible to analyse whole biological samples at the level of genome, transcriptome, proteome, metabolome and interactome after initial characterizing and phenotypic with advancement of molecular techniques. Genetic engineering techniques can be expected to make a major contribution to develop eco-friendly practices to reduce chemical burden and agriculture-based product production.

3.3 Human-Microbe Interaction

Like plant, microbes also live in constant association with human but more complex association than plants. The microbes which belonging to eukaryotes, archaea, bacteria and viruses live in surfaces, in cavities and within the cells of the human body. Therefore, this is very important for the humans to have clear information of our metagenomics to understand human biology in terms of microbial association and their role in health and disease. NIH (National Institute of health, USA) established

a project for study in human metagenomics in 2008, and it reveals some important human body parts like the oral cavity, gut, skin and vagina constantly associated with microbes (Huttenhower et al. 2012). The aim of the project is to identify and characterize the human-microbe association in order to incorporate in research and study various areas of human biology including different population, age, microbial disease, nutrition, genetics and environment. The identification and characterization of microbial community who live in the mentioned human body parts is necessary to know which is beneficial and which contributes in spreading disease. There are already ongoing many researches to interpret the role of different microbiota and their role in the skin, oral cavity, gastrointestinal tract and vagina. The microbes inside the human body are dynamic in nature and able to change the physiology of humans through taking the nutrition from the body and decreasing the immunity which finally leads to development of several kinds of diseases (Gordon et al. 2005).

3.3.1 Gut Metagenomics

Most of the human-related microbes living inside the gut play a very crucial role to nutritional metabolism, which helps physiology performance. The energy harvesting from the food only possible by microbes which are living inside human gut, any kind of disturbance of these gut microbial community leads to disease like obesity or bowel diseases (Qin et al. 2010). Subsequently, it is very important to recognize and decode the content, diversity and functioning of these gut microbes in order to good health and performance of human being. There are two kind of bacterial categories belonging to gut microbiota; first is known as *Bacteroidetes* and second *Firmicutes* revealed by 16S ribosomal sequencing on the basis of phylogenetic information (Eckburg et al. 2005). In reference to gut metagenomics, there were two important projects done, the European project, MetaHIT (Qin et al. 2010), and the American Human Microbiome Project (Huttenhower et al. 2012), which provided all reference to gene catalogue. The European project consortium, first time reveals that 3.3 million nonredundant genes inside the gut for examining foecal samples from 124 European persons. Unexpectedly, MetaHIT project also found that microbial gene set was 150 times bigger than the human gene complement, and it also concluded 99% of the genes were bacterial community and only 1% belong to other microbes (Qin et al. 2010).

3.3.2 Oral Metagenomics

The oral cavity of human anchorages the second most after gastrointestinal tract in terms of richness the microbial community. It is well known that the human oral cavity is occupied with mainly so many bacterial species; some of them are helpful, and the rest are causing oral diseases. The oral friendly bacteria live commensal and required to keep equilibrium in the mouth ecosystem but disease causing bacterial dental caries and periodontal disease (Marsh et al. 2010). There is database

dedicated to human oral metagenomics named as expanded Human Oral Microbiome Database (eHOMD) which contains all the microbial information of the oral cavity. According to this database, human oral cavity contains around 772 prokaryotic species, 70% of them are cultivable and the rest 30% uncultivable. The healthy oral bacteria are categorized into six broad phyla, which are *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Bacteroidetes* and *Spirochaetes* constituting 96% of the total oral bacteria on the basis of 16S rDNA profiling (Verma et al. 2018). Another database based on phylogenetically curated 16S rDNA of human oral microbiota called CORE includes many taxa, and accurate identification of sequence data is essential for studies of these communities. This database contains a comprehensive and less redundant number of the bacteria which are associated with the human oral cavity.

3.4 Insect-Microbe Metagenomics

As like plants, the microbial community are also stimuluses several aspects of insects' life, and this statement is gaining increasing strength, as research demonstrates it daily. Insect-associated microbial communities are attracting increasing interest, mainly because of their ecological and economical importance. Microorganisms have been investigated for the effects on their host partner, by directly mediating interactions with other species or indirectly by influencing the host genetic diversity, with effects visible at community level (Ferrari and Vavre, 2011). In plant-insect interaction, microorganisms play a very crucial role to counteract plant defence system which is very helpful for insects. The microbes live in association with insect also protect them from their enemy, support them to flourish on nutritionally marginal and help the reproductive system in the case of mutual relationship between insect and microbes (Ferrari and Vavre 2011). Both metagenomics and metatranscriptomics boosted the study of insect-microbe relationship in current scenario.

The first metagenomic study was published in the area of insect-microbes in 2016, which reconstructed biological communities on the basis of (Monteiro et al. 2016) taxonomy using a short nucleotide fragment called barcode (e.g. 16S, 18S, ITS, COI) as proxy for identification. Both metagenomic and metabarcoding approaches are useful to qualitatively evaluate the diversity of organisms in a sample but also to inform on the relative taxonomical abundance and on the presence of specific genes in that sample. However, these approaches just tell us who is in there (taxonomic reconstruction) and what is doing (gene identification).

3.5 Soil Microbial Metagenomics

The soil has supreme diversity of microbes and potentially persistent consideration of industrial, agricultural and environmental functions. As compare to other microbial sources, soil contains very diverse microbial community especially in case of prokaryotes. The research showed that ten billion microorganisms and thousands of

Table 3.1 Important project of soil taxonomic and functional diversity of microbes

No.	Name of the project	Source
1.	The Earth Microbiome Project	Gilbert et al. (2010)
2.	TerraGenome	Vogel et al. (2009)
3.	The Brazilian Microbiome Project	Pylro et al. (2014)
4.	The China Soil Microbiome Initiative	http://english.issas.cas.cn/
5.	EcoFINDERS	http://ecofinders.dmu.dk/
6.	MicroBlitz	http://www.microblitz.com.au/

different species are available in just 1 g of soil (Knietsch et al. 2003). The current advances in the area of molecular biology techniques, i.e. metagenomics boosting the research on the area of soil microbial diversity to reveal the opportunity and scale of relevant question related to this composite habitat their metabolic important inside soil community. The NGS (new generation sequencing) technologies have also provided support to illustrate and quantify soil microbial diversity (Nesme et al. 2016). There were some important projects done to explore the soil taxonomic and functional diversity of microbes at large scale given in Table 3.1.

3.6 Metagenomic Resources

Various metagenomic resources are available in public domain for research and education. A brief summary of metagenomic tools and databases is summarized in Table 3.2.

3.7 Conclusion and Future Perspective

In present, various modern approaches are common in microbial research to reveal various uncultivable microbes in biological system. Apart from these molecular and computational progresses, most of the analyses highly depended on known reference sequences and databases. Microbial genomes are very dynamic and change rapidly which is not enough to serve all time reference set for microbial ecology and evolution studies. So, enrichment and update of reference sequences is one of the major tasks in metagenomic research. In present, a vast majority of data have been generated from various long-term projects for large-scale meta-analysis and interpretation. But development of integrated research platform and use for hidden knowledge and law discovery of microbial ecosystem will be another challenge. The species isolation and identification methods have improved significantly to characterize biological system but are still challenging at large-scale studies. Nowadays, application of microbiome for sustainable life on earth is one of the hotspot areas of microbial research in biological science. In various research studies, microbial influence for human, plant and animal fitness has been witnessed and suggested that high microbial diversity may act as an outer layer of immunity which

Table 3.2 Important metagenomic database and their description with link

Name of database	Description	www/open source	Availability
Metagenomic genome resource			
IMG	Integrated Microbial Genomes and Microbiome. Repository of 33,116 genome datasets and 4615 microbiome datasets	*Yes/Yes*	https://img.jgi.doe.gov/
MGDB	Microbial genome database with 4742 genomes	*Yes/Yes*	http://mbgd.genome.ad.jp/
ENSEMBL	Access to over 40,000 bacterial genomes	*Yes/Yes*	http://bacteria.ensembl.org/index.html
RefSeq (microbial)	Archaeal and bacterial repository at NCBI Reference Sequence	*Yes/Yes*	https://www.ncbi.nlm.nih.gov/refseq/
Catalogue and media database			
MediaDB	Collects chemically defined growth media from literature sources for fully sequenced organisms	*Yes/Yes*	https://mediadb.systemsbiology.net/defined_media/
MBMDB	Microbiology media database with the information of category, solid and liquid ingredients, method of preparation and uses	*Yes/Yes*	http://bioinfodb.com/frontend/index.php
KOMODO	A platform for recommending microbial media	*Yes/Yes*	http://komodo.modelseed.org/
WDCM	Worldwide microbial culture collection	*Yes/Yes*	http://www.wfcc.info/ccinfo/home/
GCM	Global catalogue of microbes	*Yes/Yes*	http://gcm.wfcc.info/
Microarrays and gene expression database			
(M3D)	Many microbe microarrays database	*Yes/Yes*	http://m3d.mssm.edu/
BμG@Sbase	Microarray datasets for microbial gene expression	*Yes/Yes*	http://bugs.sgul.ac.uk/bugsbase/tabs/experiment.php
COLOMBOS	Collection of bacterial gene expression compendium	*Yes/Yes*	http://www.colombos.net/
Microbeonline	Repository of 3707 genomes, gene expression data for 113 organisms	*Yes/Yes*	http://www.microbesonline.org/
Taxonomic, functional annotation and comparative genomics			
POGO	Database of pairwise comparisons of genomes and orthologous genes	*Yes/Yes*	http://pogo.ece.drexel.edu/about.php
MicroScope	Microbial genome annotation and analysis platform	*Yes/Yes*	https://www.genoscope.cns.fr/agc/microscope/home/

Artemis	Genome browser and annotation tool, visualization of sequence features, NGS data analyses		http://www.sanger.ac.uk/science/tools/artemis
AGeS	A software system for microbial genome sequence annotation	*Yes/Yes*	http://www.bhsai.org/ages.html
NMPDR	National Microbial Pathogen Data Resource for annotation, comparative genomics with an emphasis on the food-borne pathogens	*Yes/Yes*	http://www.nmpdr.org/FIG/wiki/view.cgi
GenoList	An integrated environment for comparative analysis of microbial genomes		http://genolist.pasteur.fr/
MetaPathways	A pipeline for taxonomic and functional annotation from environmental sequence information	*Yes/Yes*	http://hallam.microbiology.ubc.ca/MetaPathways/
ShotgunFunctionalizeR	An R-package for functional comparison of metagenomes	*Yes/Yes*	http://shotgun.math.chalmers.se/
MG-RAST	Automated analysis platform for metagenomes based on sequence data	*Yes/Yes*	http://metagenomics.anl.gov/
MEGAN	A comprehensive toolbox for interactively analysing microbiome data	*Yes/Yes*	http://ab.inf.uni-tuebingen.de/software/megan6/welcome/
Specialized resources for microbial studies			
MiST	Microbial Signal Transduction in complete microbial genomes database	*Yes/Yes*	http://mistdb.com/
mVOC	Microbial volatile organic compounds database	*Yes/Yes*	http://bioinformatics.charite.de/mvoc/
SIDDBASE	Stress-induced DNA duplex destabilization profiles of complete microbial genomes	*Yes/Yes*	http://benham.genomecenter.ucdavis.edu/
sRNAMap	Small regulatory RNA in microbial genomes		http://srnamap.mbc.nctu.edu.tw/
VFDB	Virulence Factors Database for microbial virulence factors	*Yes/Yes*	http://www.mgc.ac.cn/VFs/main.htm
STRING	Gene and protein interaction network tool	*Yes/Yes*	http://string-db.org/
HAMAP	High-quality Automated and Manual Annotation of microbial Proteomes		http://hamap.expasy.org/
2D-PAGE	Proteome database system for microbial research		http://www.mpiib-berlin.mpg.de/2D-PAGE/
ClusterMine360	Microbial PKS/NRPS biosynthesis		http://www.clustermine360.ca/

(continued)

Table 3.2 (continued)

Name of database	Description	www/open source	Availability
DESM	Microbial knowledge exploration systems	*Yes/Yes*	http://www.cbrc.kaust.edu.sa/desm/home/index.php
MICdb3.0	A comprehensive resource of microsatellite repeats from prokaryotic genomes		http://micas.cdfd.org.in/
Metabolic modelling tool and databases			
CellDesigner	Metabolic pathway reconstruction and simulation	*Yes/Yes*	http://www.celldesigner.org/
E-zyme	Prediction of EC numbers from chemical transformation pattern	*Yes/Yes*	http://www.genome.jp/tools/e-zyme/
Triton	Tool for enzyme engineering	*Yes/Yes*	www.ncbr.muni.cz/triton/
ECMDB	*E. coli* Metabolome Database	*Yes/Yes*	http://ecmdb.ca/
MicrobesFlux	A web platform for genome reconstruction and constraint based modelling	*Yes/Yes*	http://www.microbesflux.org/
MetaCyc	MetaCyc metabolic pathway database	*Yes/Yes*	http://metacyc.org/
MetaBioMe	Data mining engine for known commercially useful enzymes (CUEs) in metagenomic datasets and genomes	*Yes/Yes*	http://metasystems.riken.jp/metabiome/
Metabolome searcher	HTS tool for metabolite identification and metabolic pathway mapping directly from mass spectrometry and metabolites		http://procyc.westcent.usu.edu/cgi-bin/MetaboSearcher.cgi
ProCyc	An open resource for the study of metabolic capabilities in microorganisms from food, environment and specific pathogens from these sources	*Yes/Yes*	http://procyc.westcent.usu.edu:1555/
MEMOSys	Bioinformatics platform for genome-scale metabolic models	*Yes/Yes*	http://icbi.at/software/memosys/memosys.shtml
EAWAG-BBD	Microbial biocatalytic reactions and biodegradation pathways	*Yes/Yes*	http://eawag-bbd.ethz.ch/
Desharky	Microbial biodegradation to host metabolites		http://soft.synth-bio.org/desharky.html
PathPred	Microbial biodegradation	*Yes/Yes*	http://www.genome.jp/tools-bin/pathpred/pathpred.cgi
CRAFT	Chemical reactivity and fate tool		https://www.mn-am.com/products/craft
	Biodegradation of aerobic bacteria		

EAWAG-BBD/PPS/BPT	Biodegradation information of aerobic/anaerobic bacteria	*Yes/Yes*	http://eawag-bbd.ethz.ch/
MetaboleExpert	Biodegradation by plants and animals		http://www.compudrug.com/metabolexpert
ModelSEED	Microbial and plant metabolic modelling	*Yes/Yes*	http://modelseed.org/
COBRAToolBox	Constraint-based modelling; MATLAB and Python	*Yes/Yes*	http://opencobra.github.io/cobratoolbox/
OptFlux	Tool for metabolic engineering	*Yes/Yes*	http://www.optflux.org/

can help to handle biotic and abiotic stressors in changing climatic conditions. A strategic research is required to balance human-microbe and plant-microbe interaction to gain mutual benefit and reduce evolutionary arm race between pathogenic microbes and hosts.

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4 Applications of Microarray-Based Technologies in Identifying Disease-Associated Single Nucleotide Variations

Sartaj Khurana, Sudeep Bose, and Dhruv Kumar

Abstract

The analysis of a multitude of genes in one shot has been made possible with the introduction of microarrays to the scientific community. Microarrays are microscopic slides that are printed with thousands of tiny spots with each spot containing a specific nucleotide (known DNA). These nucleotides act as probes to detect the expression of the desired gene (mRNA). With growing scientific knowledge over the years, microarrays have found applications in a plethora of research specializations such as gene discovery, mutational analysis, detection of single nucleotide polymorphisms, identification and detection of microorganisms, and detection of clinical conditions such as cancer, heart diseases, neurological disorders, etc. Detection and diagnosis of such clinical conditions are now relatively easy with techniques such as microarrays, and timely therapeutic intervention is now no more a farfetched dream. Microarrays are these days being used to their full potential as elucidated by a variety of studies suggesting that the utility of microarrays will continue to grow in the forthcoming years as viable detection and identification methods.

Keywords

Microarray · SNPs · CNVs · Gene expression · Mutational analysis

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4.1 Introduction

4.1.1 DNA Microarrays

Molecular biology-based research has seen immense evolvement through the development of a multitude of technologies. Traditional methods do not allow analysis of a large number of samples/genes which is why microarray-based techniques have been brought into picture. Hybridization of mRNA onto a DNA template is the characteristic of a typical microarray. The expression of the gene of interest is measured by the amount of mRNA bound to the template. There are different variants of microarrays, one of which is a DNA-based microarray. Often referred to as a nucleic acid array, this technology involves the use of specific DNA sequences that hybridize covalently or non-covalently to the surface. The intensity of the fluorescence at each of the spots in the array post-hybridization corresponds to the expression of the respective gene in that particular spot in the array (Inaoka et al. 2015). There are different types of DNA microarrays based on the distinguishing characteristics such as the nature of the probe or the specific method used for target detection (Miller and Tang 2009), for instance, printed-type microarrays which are so named because of printing of the probes onto a glass surface due to economic reasons as well as stability of glass at high temperatures and negligible background fluorescence (Cheung et al. 1999). Other types include in situ synthesized types, high-density bead arrays, electronic microarrays, etc. Microarray technology has well-established roles in clinical microbiology (Arenas et al. 2019; Dey et al. 2019), typing of human papillomaviruses (Moreas et al. 2014), gene expression profiling (Yao et al. 2019), and cancer research (Inaoka et al. 2015). Even with these major breakthroughs, microarray technologies have still not been used to their utmost potential. This chapter emphasizes the involvement of microarrays and its applications in areas of cellular and molecular biology and its use for diagnostic and therapeutic purposes.

4.1.1.1 Gene Expression Analysis

DNA microarrays are microscopic platforms printed with thousands of tiny spots in specific positions. Each of these spots contains a known DNA sequence or gene that act as probes to detect gene expression. DNA microarrays have the ability to simultaneously analyze the expression levels of a plethora of genes for multiple purposes such as (i) identification of diagnostic and prognostic markers, (ii) classification of diseases, (iii) monitoring the prognosis, and (iv) understanding the mechanisms behind the pathogenesis of a diseases, etc. (Tarca et al. 2006). DNA microarrays have been used to analyze the changes in the gene expression of a large number of genes in one go by fluorescently labeling the complementary DNAs (Fig. 4.1). A recent study by Prasad et al. analyzed expression in the breast cancer-1 (BRCA-1) gene from exon 11 using a portable fluorescence microarray-based imaging system which was connected to a smartphone (Prasad et al. 2018). Microarrays have also been well used in the analysis of genome-scale DNA methylation in a large cohort of colorectal cancer subjects. The study by Fennell et al. revealed five subtypes of colorectal cancer that were clinically and molecularly distinct from each other

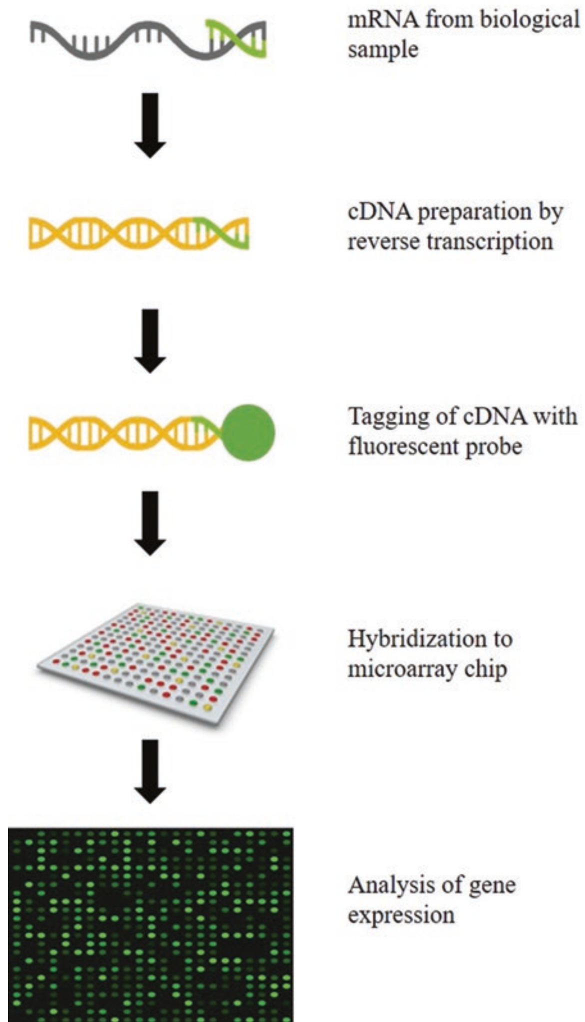

Fig. 4.1 Schematic diagram representing the use of microarrays for the analysis of gene expression

(Fennell et al. 2019). Another important application of microarrays in gene expression analysis was seen very recently in a German study led by Koschmieder which threw light on the microarray-detected expression levels of interferon-stimulated gene (ISG) in CML subjects (Schubert et al. 2019). In microorganisms such as *Mycobacterium*, microarrays have been seen to be extremely vital in assessing parameters such as antibiotic exposure, nutrient starvation, oxygen limitations, etc. (Bacon et al. 2004; Briffotaux et al. 2019).

4.1.1.2 Transcription Factor Binding Analysis

Microarrays accompanied with ChIP assays have been used to identify the binding sites of transcription factors (TFs) of interest. The methodology involves the

cross-linking of TFs to DNA using formaldehyde followed by fragmentation of the DNA. The TF-DNA complex is purified using an antibody or tagging the TF with a peptide and purified using affinity chromatography. The peptide tag can be a FLAG-, HIS-, MYC, or HA. Once purified, the TF-DNA complex breaks allowing the DNA to be released from the TF followed by amplification, labeling, and hybridization onto the microarray surface (Bumgarner 2013). Protein-binding microarrays, abbreviated as PBMs, are techniques that have been well established to assess the in vitro binding of the target proteins to DNA. This technique involves the binding of a protein to a double-stranded DNA microarray followed by quantifying the amount of protein tagged to the DNA using a fluorescent antibody (Andrilenas et al. 2015). PBMs have been used recently to determine the binding specificity of TFs such as retinoid X receptors (RXR) as elucidated by Reitzel et al. (2018). The study elucidated that the TF binding sites were broadly conserved (approx. 85% similarity) pointing toward an evolutionary drift from *T. adhaerens* to humans.

4.1.1.3 Genotyping Analysis

DNA microarrays have recently been used for applications such as genotyping of single nucleotide polymorphisms (SNPs) (Arbitrio et al. 2016) and detection of gene copy number (CN) (Borlot et al. 2017). A recent study by Kumar et al. from Southern India identified 29 SNPs in triple-negative breast cancer (TNBC). The study was aimed at identifying markers that might be viable contenders in predicting TNBC (Aravind Kumar et al. 2018).

One of the variants of microarrays is chromosomal microarray (CMA) that encompasses comparative hybridization (CGH) and SNP array. CGH is usually designed for the purpose of copy number variant (CNV) detection using any of the two – either a bacterial artificial chromosome (BAC) or oligonucleotide probes. Affymetrix launched CytoScan HD array platform (Scionti et al. 2018) that comprises of almost two million CN markers and 750,000 SNPs that can be genotyped. This platform has seen applications in neurological disorders such as cerebral palsy (Zarrei et al. 2018), identity disorders (ID) (Wang et al. 2019), and William-Beuren syndrome (Fan et al. 2016). Such microarray techniques have proved to be an edge over the other technologies such as karyotyping due to enhanced diagnostic yield and increased resolution.

4.1.1.4 Mutational Analysis

It is of utmost importance to identify the DNA variants that are capable of causing diseases, for example, cancer where detection of oncogenic mutations can prove helpful for timely diagnosis and treatment. Mutations in the KRAS, NRAS, and BRAF genes in colorectal cancer have been recently reported in people with colorectal cancer using microarray technologies (Damin et al. 2018). Not only cancer but reports of mutations in citrullinemia subjects have also recently surfaced elucidating mutated ASL and SLC25A13 genes, thus expanding the mutational spectrum that underlies citrullinemia (Lin et al. 2017). Interestingly, a recent chromosomal microarray analysis identified a small, rare deletion on chromosome 12q24.31 calling it the "microdeletion syndrome" which results in

neurodevelopmental delay and behavioral issues (Palumbo et al. 2015). Similarly, microarray technologies have paved ways for researchers to identify a multitude of other mutations in clinical conditions such as autosomal dominant retinitis (Van Cauwenbergh et al. 2017), familial adenomatous polyposis (Nallamilli and Hegde 2017), and hemophilia B (Jourdy et al. 2016).

4.1.2 Applications of Microarrays

4.1.2.1 Gene Discovery

Identification of dysregulated genes has been made quite easier since the dawn of the microarray era. Genome-wide association study (GWAS) analysis was recently made use of in a Spanish study by Roca-Ayats et al. which revealed that C7ORF76 mutations may be linked with osteoporosis (Roca-Ayats et al. 2019). Another variant of DNA microarrays are cDNA microarrays which have recently been applied in many studies. For instance, de O Coelho et al. employed cDNA microarrays to identify target genes that were associated with reversion of skeletal muscle atrophy (de et al. 2019). Another study to follow the same microarray strategy was conducted by Su et al. where the team identified several differentially expressed genes with great possibility of being potential biomarker candidates in the prognosis of lung adenocarcinoma (Su et al. 2019). Such studies throw light on the prospective of microarrays and their multifaceted nature making them extremely reliable for research.

4.1.2.2 Disease Diagnosis

Effective diagnosis of diseases and disorders is vital in order to open appropriate therapeutic avenues. In the past recent years, microarrays have proved to be extremely efficient in the detection of a plethora of clinical conditions such as cancer (Peterson et al. 2015; Postovit et al. 2019), diabetes (Singh et al. 2016), congenital heart diseases (Wang et al. 2018b), allergies (Jeon et al. 2018), etc. Apart from the abovementioned diseases, microarrays have also been put to their best use in detection of neurological disorders (Sim et al. 2019), autoimmune disorders (Yeste and Quintana 2013), avian viral infections (Sultankulova et al. 2017), obesity, and type 2 diabetes (Zeng et al. 2019).

4.1.2.3 Cancer

The keyword "cancer" gives a total of 3,844,894 results with almost 1000 new additions every day on PubMed making it one of the most researched areas today. With growing studies on the pathophysiology, diagnosis, and management of cancer, proper detection is warranted. Microarray technology has been of great help in detection and diagnosis for a long time and continues to be of utmost utility even now. A 2017 study by Kim et al. made use of dendron-coated DNA microarrays in order to identify Caveolin-1 as a cell invasion gene in liver cancer cells demonstrating the potential of microarrays in diagnosis of diseases (Kim et al. 2017). Another study by Clarke et al. in 2017 threw light on the proficiency of microarrays in

diagnosis of cervical cancer and precancer using a bead-based microarray (Clarke et al. 2017). Very recently there have been multifarious evidences that revealed the importance of microarrays in cancers such as gastric cancer (Kim et al. 2018b), breast cancer (Prasad et al. 2018), bladder cancer (Kusuhara et al. 2019), lung cancer (Wang et al. 2018a), etc. The utility of microarrays has grown over the past many years and will continue to grow as viable tools for effective diagnostic purposes.

4.1.2.4 Heart Disease

Cardiovascular disorders have been a major cause of mortality worldwide since a long time. In order to curb the mortality, timely diagnosis is crucial to ensure development of appropriate treatment methods. Recent revelations have demonstrated the importance of microarrays in identification of cardiac risk factors and hence the diagnosis of heart-related diseases. A study by Maneerat et al. performed gene profiling using DNA microarrays in Thai patients with hyperlipidemia and elucidated the role of pro-platelet basic protein (PPBP) and alpha-defensin (DEFA1/DEFA3) as important biomarkers of coronary heart disease (CHD) (Maneerat et al. 2017). With the advent of CMAs, identification of chromosomal anomalies has become much easier. Congenital heart defects are chromosomal aberrations that are usually caused usually by environmental risk factors such as maternal diseases, fetal teratogens, or sometimes genetic risk factors like single gene mutations, aneuploidy, chromosomal rearrangements, etc. A recent study from China employed CMAs for the detection of chromosomal anomalies and copy number variations in fetuses with congenital heart diseases (Xia et al. 2018). A similar study was performed by Wu et al. which shed light on the role of CMAs in diagnosing children with congenital heart diseases (Wu et al. 2017). Such studies have proven that microarray techniques especially CMAs are extremely efficient in the diagnosis of heart diseases.

4.1.2.5 Neurological Disorders

With more than 600 types of diseases, neurological disorders are undoubtedly a global burden. Affecting majorly the central nervous system including the brain, spine, cranial nerves, peripheral nerves, nerve junctions, and muscles, few of the major contributors are epilepsy, Alzheimer's disease, dementia, Parkinson disease, autism, and brain tumor. The use of microarrays has been well documented in such disorders to ensure timely diagnosis. CMAs have been previously studied and proven to be valuable in diagnosis of disorders such as dysmorphia, developmental delay or intellectual disability (DD/ID), autism, and congenital anomalies (Sansovic et al. 2017). A similar study conducted by Lee et al. diagnosed DD/ID using CMA in a Korean population identifying a total of five rare chromosomal deletions on 2p21p16.3, 3p21.31, 10p11.22, 14q24.2, and 21q22.13 indicating the utility of CMA in disease diagnosis (Lee et al. 2018). Recently, reports have surfaced that have elucidated the applications of CMA in diagnosing disorders like epilepsy (Peycheva et al. 2018), fetal cerebral ventriculomegaly (Peng et al. 2018), Parkinson disease (Williams et al. 2018), and even rare disorders like Williams syndrome (Xia et al. 2019).

4.1.2.6 Microbial Detection and Identification

Microarrays have made it simpler for researchers to detect pathogens that are capable of causing infections, and early detection paves way for prompt treatment. DNA microarrays have been well applied in detecting pathogens such as viruses (Martinez et al. 2015; Liu et al. 2017; Kim et al. 2018a), bacteria (Bannister et al. 2018; Song et al. 2018), fungi (Cao et al. 2018), and protozoa (Chen et al. 2016). Not only in humans, microarrays have also found applications in detection of pathogens in plants. For instance, a study published in *Phytopathology* laid emphasis on the use of DNA microarrays in detection of multiple pathogen species responsible for the causal of sugar beet root rot diseases (Liebe et al. 2016). Similarly, a 2018 study based out of Russia identified a total of six fungal pathogenic species in potato plants revealing great potential that microarrays hold (Nikitin et al. 2018).

4.1.2.7 Clinical Microbiology

Science has been consistently making advances since the birth of the microarray technology as microarrays have been exploited well to identify several pathogenic species and investigate the differential expression of the genes underlying the pathogenic mechanisms. For instance, Zika virus (ZIKV) infections are rare and are spread mostly by the bite of *Aedes* mosquitos. Consequently, these infections are known to cause serious birth defects and may in some cases trigger paralysis. A recent study published in *Scientific Reports* made use of microarray techniques to diagnose Zika virus infections revealing the presence of ZIKV in 13 of 42 tested sera (Hansen et al. 2019). Microarrays are consistently achieving new heights in diagnostics. A study from Spain reported a portable point of care (PoC) microarray device capable of detecting *E. coli* bacteria for efficient and accurate clinical diagnosis (Dey et al. 2019). Intriguingly, with the advent of science and technical know-how, a Brazilian group led by Moretti developed a DNA microarray platform which helped them identify pathogenic fungi strains in blood culture bottles (Sturaro et al. 2018). With growing evidences about the involvement of microarray techniques in clinical microbiology like identification of bacterial genes promoting human diseases (Young et al. 2019) or detection of genes playing a role in lipid metabolism and weight regulation (Valsesia et al. 2019), it would be correct to say that microarrays and techniques alike are have proven to be extremely handy.

4.1.2.8 Drug Discovery

DNA microarrays have the ability to measure the expression patterns of multifarious genes at once and further providing aid in identifying appropriate targets for therapeutic intervention. As mentioned earlier, cancer is one of the most addressed areas of research and requires appropriate and prompt therapeutic strategies. Interestingly, a Chinese team identified potential gene signatures responsible for development and progression of basal-like breast cancer (BLBC) which is apparently the most aggressive subtype of breast cancer. The study identified 40 upregulated and 21 downregulated differentially expressed genes using microarray technology paving way for on-point potential treatment strategies for BLBC subjects (Yang et al. 2019). Microarray-based strategies such as GWAS have been

widely used for identification of genes for therapeutic purposes in clinical conditions like intraocular pressure (IOP) that poses as a major risk factor for glaucoma (Huang et al. 2019), rheumatoid arthritis (Ferreiro-Iglesias et al. 2019), Alzheimer's disease (Han et al. 2019), chronic obstructive pulmonary disease (COPD) (Wain et al. 2017), obesity (Nizamuddin et al. 2015), and cardiovascular diseases (Folkersen et al. 2017).

4.1.3 Discussion and Conclusion

Since the dawn of the microarray era, it has been relatively easier for scientists to analyze a large amount of huge amount of data in one go. Microarrays are microscopic slides that are printed with thousands of tiny spots with each spot containing a specific nucleotide (known DNA). These nucleotides act as probes to detect the expression of the desired gene (mRNA) (Fig. 4.1). mRNA from biological samples are reverse transcribed to cDNA which are then labeled with fluorescent dyes and bound to the microarray slide for detection. The gene expression is analyzed using the intensity of the fluorescence emitted. Over the past many years, microarrays have found themselves to be applicable in a plethora of research specializations such as gene discovery, mutational analysis, detection of single nucleotide polymorphisms, identification and detection of microorganisms, and detection of clinical conditions such as cancer, heart diseases, neurological disorders, etc. (Fig. 4.2). Applications of microarrays in disease diagnosis have made it relatively easier for researchers to detect clinical conditions such that appropriate therapeutic measures can be undertaken timely. Microarrays are these days being used to their full potential as elucidated by a variety of studies suggesting that the utility of microarrays will continue to grow in the forthcoming years as viable detection and identification methods.

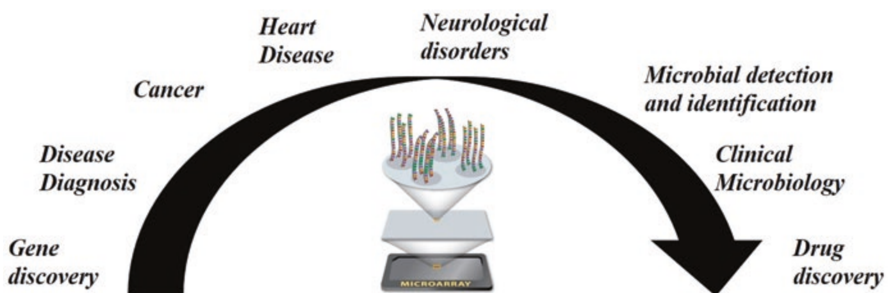

Fig. 4.2 Applications of microarrays in cellular and molecular biology and its use for diagnostic and therapeutic purposes of several diseases

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Impact of Microbial Genomics Approaches for Novel Antibiotic Target

5

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Abstract

Infectious diseases are life-threatening and may lead to high mortality and morbidity rates. The existing danger of an increase and spread of multidrug resistance pathogens is a global concern. Therefore, the designing of novel antibiotics and vaccine to control and eliminate the disease is an utmost requirement. Traditional approaches for screening vaccine and drug targets are time-consuming and have been unsuccessful in controlling the spread of infectious diseases due to several reasons such as altered antigenic diversity, altered virulence potential, and antimicrobial resistance in the infectious agent population. To overcome this problem, there has been a paradigm shift from the conventional to microbial genomics approaches, as the availability of complete genome sequence of pathogenic microorganisms and multiple isolates of the same species provides a wealth of information on nearly all the potential drug targets. Microbial genomics approaches open up new avenues to pursuit novel antimicrobial agents that are highly conserved in a range of microbes, essential for the survival of pathogens and absent in humans. In this chapter, we present an overview of the microbial genomics approaches such as pan-genomics, comparative genomics, functional

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genomics, structural genomics, transcriptomics, and proteomics used in the discovery and development of novel antibiotics.

Keywords

Microbial genomics · Antibiotics and vaccine target · Pan-genomics · Structural genomics · Transcriptomics · Proteomics

5.1 Introduction

Earlier, the infections caused by microbes had been a massive problem, but in the year 1940s, it was resolved by the introduction of antibiotics. With this advancement in treatment protocol, in the 1960s, it was stated that the danger of microbial infection is no more a problem and the microbes could be successfully defeated. However, unfortunately over the last decades, the microbes acquired resistance toward antibiotics leading to a broader health concern. Therefore, nowadays microbial antibiotic resistance is an emergent and hazardous issue worldwide, and this necessitates novel antibiotics to combat microbial infection.

Development of effective antibiotics and vaccines against infectious disease has a major impact on health globally. The increasing antibiotic resistance and varied antigenic diversity among the pathogens are raising severe concern for the future pandemic. A recent report of the Centers for Disease Control and Prevention (CDC) on "antibiotic resistance: a global threat" showed that only in the USA, every year approximately 2 million people are infected by antibiotic-resistant strains, accounting for nearly 23,000 deaths (CDC 2018; https://www.cdc.gov/features/antibiotic-resistance-global/index.html). In this report, the negative impact of antimicrobial resistance on economy was also predicted with an expected loss of around $100 trillion by the year 2050. These estimation prioritize our action toward finding essential targets and mechanisms for the development of novel vaccines and drugs.

Conventional approaches have proven insufficient to study pathogens because of the complex mechanism of pathogenesis, varied antigenic diversity, as well as lack of a suitable animal model of infection. The arrival of the genomic era has a great impact on the development of vaccine and antibiotics. Microbial genomics data from genome, transcriptome, proteome, immunome, or structural genome provides a wealth of information about the different pathogens that seems to be sufficient for rapid development of novel vaccine and therapeutic and to limit the spread of infection. Therefore, the present chapter aims to provide a comprehensive overview of microbial genomics approaches and their significance in the development of novel vaccines and antibiotics.

5.2 Essential Criteria of Vaccines and Therapeutic Targets

The identification of drug and vaccine targets can be achieved by using various approaches such as the comparative and structural genome, transcriptome, proteome, and immunome. These approaches can be applied in several combinations based on nature of the pathogen under study. However, it is necessary to consider the following basic criteria while selecting the potential targets: (i) target should be specific and highly selective against the microbe rather than host and also active against a broad spectrum of pathogens, (ii) target should be essential for the growth and survival of pathogens at the time of infection, (iii) target should be expressed or easily accessible to the host immune system during the course of infection, and (iv) some prior information about the function of target is necessary so that high-throughput assays can be performed. Identification of new potential targets can be initiated by using the criteria mentioned above which would be helpful in finding out the successful targets.

5.3 Microbial Genomics Approaches

Since the completion of the first bacterial genome sequence of *Haemophilus influenzae* in 1995, the idea for the development of vaccine and therapeutic approaches shifted from conventional approaches to microbial genome-based approaches. Several microbial genomics approaches such as genomics, pan-genomics, comparative genomics, functional genomics, structural genomics, transcriptomics, and proteomics have been utilized for this purpose. The schematic representation of several important microbial genomics approaches has been shown in Fig. 5.1. In summary, in silico screening of the entire genome sequence of the pathogen (genomics) provides complete information about the genetic repertoire of antigens and drug targets. Pan-genomics helps in the identification of conserved antigens and thereby in the identification of potential drug targets through the investigation of genetic material from numerous organisms of single species. Next, it is essential to compare the genetic material of pathogenic and nonpathogenic organisms of single species. This is crucial for identifying antigens or targets that are present in pathogenic strains but absent in nonpathogenic strains. Transcriptomics and proteomics aim to recognize the set of RNA transcripts and proteins expressed by an organism under a specified circumstance and in specific cellular location. Further, the analysis of genes and proteins array would help to understand the survival of an organism under a specific condition (functional genomics). Some interesting field of study emphasizes the identification of protein arrays or epitopes that interact with the host immune system and the possible mechanisms of their interaction (immunomics). Analysis of the three-dimensional structure of proteins of an organism and the process of interaction with antibody and therapeutics (structural genomics) can provide a clear idea about the biological phenomena and potentiality of a novel drug. Following this the vaccinomics approach enables the monitoring of the mode of response of the human immune system to a vaccine or drug. Finally, if the identified targets show

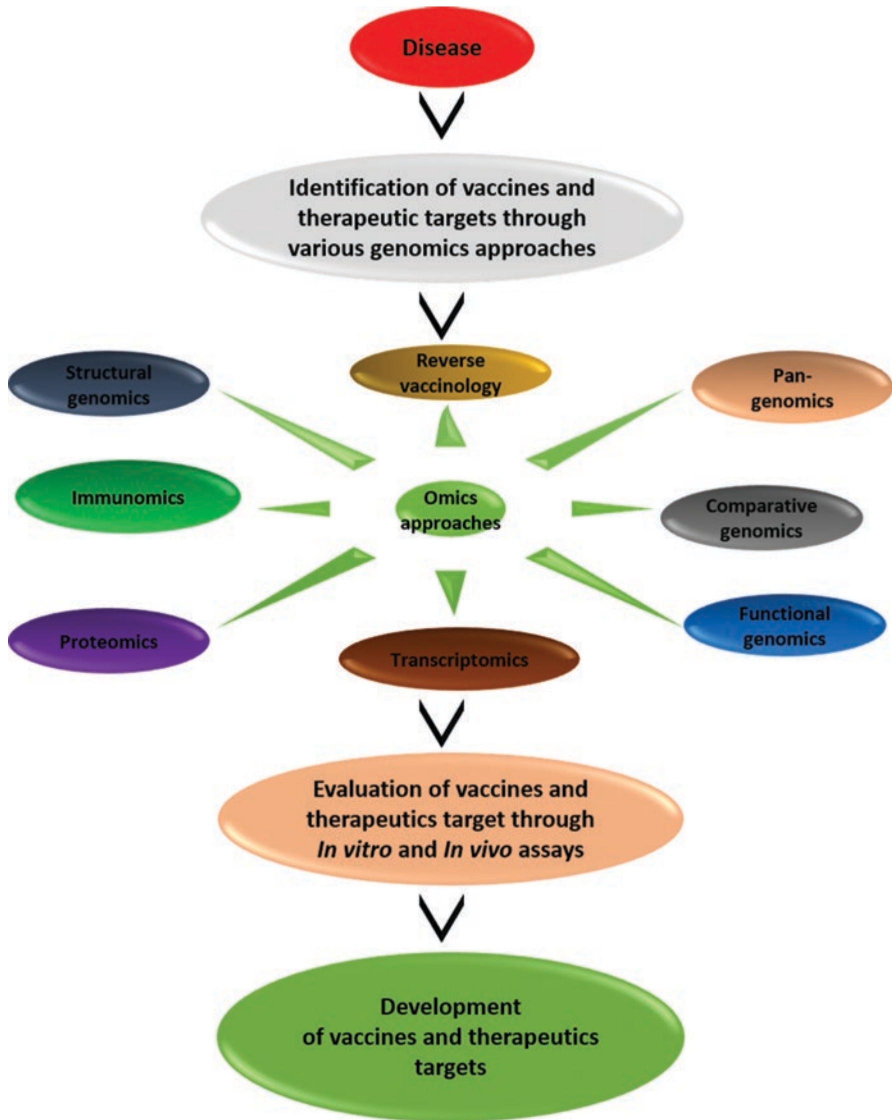

Fig. 5.1 Schematic representation of microbial genomics approaches for the development of vaccines and therapeutics

protection against disease and have low risk vs benefit ratio for humans, they are subjected for clinical trials, and then clinically tested vaccine and therapeutic targets can be licensed for use. In Table 5.1, a brief description of various microbial genomics approaches along with their limitations has been presented.

Here, we are summarizing genomics, pan-genomics, comparative genomics, functional genomics, structural genomics, transcriptomics, and proteomics-based

Table 5.1 Overview of microbial genomics approaches for the development of vaccines and therapeutics

Genomic approaches	Description	Limitations
Reverse vaccinology/ genomics	Identification of surface-exposed proteins as vaccine/therapeutic targets that targets organisms has the potential to be express	Nonprotein antigens such as lipids (glycolipid, phospholipid) and polysaccharides and posttranslational modification (glycosylation, methylation, mannosylation) cannot be identified (Seib et al. 2009)
		Requirement of high-throughput cloning and protein expression
Pan-genomics	Identification of conserved targets through analysis of genetic material of several organisms of single species	Requirement of the genomic sequence of several strains of same species
		Same limitation as described above
Comparative genomics	Identification of genes that are present in pathogenic strain but absent in nonpathogenic strain through analysis of genetic material of different strains of the same species	Same limitations as described for the above two approaches
Transcriptomics	Identification of global changes in gene expression through analysis of RNA transcripts level of an organisms under specified conditions	Large quantity of mRNA is required for in vivo studies
		Difficulty to make probes because bacterial mRNA is unstable
		There is no direct correlation between transcription product (i.e., mRNA) and translational product (i.e., protein)
Functional genomics	Evaluate the function of genes and proteins to identify the genes that are essential for survival and pathogenesis of an organism under specific conditions	Pathogen should be naturally competent; otherwise, it will not be able to accept the transposon (Seib et al. 2009)
Proteomics	Identification of the entire set of proteins expressed by an organism under specified conditions	Proteins should not be in low abundance
		Those proteins, which are expressed in in vivo conditions but not capable to express in in vitro conditions, may not be able to detect
		Low-solubility proteins also may not be able to identify
Immunomics	Evaluate the complete set of proteins that interact with the host immune system to identify the B-cell and T-cell epitopes	Difficult to predict the B-cell antigenic determinant
		Only configurational epitopes can be detected. Chances to detect conformational epitopes is very low (Seib et al. 2009)
		Potential bias against non-displayed sequences
Structural genomics	Identification of the three-dimensional structure of proteins expressed by an organism and how they interact with drugs or antibodies	Inadequate apprehension of antigenic determinants of immunogenicity (Seib et al. 2009)
		Poor understanding of the structure-function relationship

microbial genomics approaches in the context of identification and characterization of potential targets as a drug or vaccine candidate.

5.3.1 Reverse Vaccinology/Genomics

Reverse vaccinology is the in silico screening of the pathogen genome to find out the repertoire of antigens/drug targets that are expressed by the organism. By using various bioinformatics tools, it is possible to predict the ORFs of all the genes that are exposed or secreted on the surface of pathogen. Genes which are uniquely present in a certain pathogen can be selected for in vitro and in vivo studies. This involves a few critical experimental steps like gene cloning and expression, protein purification, and then selection of the potential candidate (Grandi 2001). One of the best examples of reverse vaccinology approach is serogroup B *Neisseria meningitidis* (MenB) project. In this project, numerous novel vaccine candidates were determined in a period of 18 months, and it outnumbered the discovery made in 40 years of conventional vaccinology (Pizza et al. 2000). In the analysis of MC58 strain genome (belongs to MenB), 570 ORFs out of 2158 ORFs were predicted to encode either surface exposed or secreted (Pizza et al. 2000). Antigen sorting was continued based on handful criteria which include the identification of the ability of antigens to be cloned and expressed in *Escherichia coli* as recombinant proteins (350 candidates) followed by the validation of the antigen exposed on the cell surface (91 candidates) by ELISA and flow cytometry. To confer protective immunity, the ability of induced antibodies (28 candidates) was measured by serum bactericidal assay or passive protection in infant rat. Further, screening was performed to identify the conservation of potential antigens in a panel of diverse meningococcal strains especially pathogenic strains of MenB (Rappuoli 2008; Giuliani et al. 2006). Using this methodology it was possible to identify five antigens, (i) genome-derived *Neisseria* antigens 1870 (GNA1870; which is factor H-binding protein [fHBP]), (ii) GNA1994 (which is NadA), (iii) GNA213, (iv) GNA1030, and (v) GNA2091. It also enabled the classification of outer membrane vesicles (OMV) from the New Zealand MeNZB vaccine strain that contains the immunogen PorA (Martin et al. 2006) and has been combined to form the Novartis MenB vaccine which entered the phase III clinical trials in 2008 (Rappuoli 2008; Giuliani et al. 2006).

5.3.2 Comparative Genomics

This approach is used to compare the pathogenic and nonpathogenic strains of the same species in order to identify the unique genes that are only present in pathogenic strains but absent in nonpathogenic strains. Those unique genes that are involved in pathogenesis and virulence of organisms might be the potential target for the development of vaccine and therapeutics (Bhagwat and Bhagwat 2008). Rasko et al. (2008) identified some genes that are present only in pathogenic strains of *E. coli* but absent in commensal strains during comparison of up

to 17 commensal and pathogenic strains of *E. coli*. With the rapid advancement in sequencing technology and bioinformatics, an exponential growth in genome sequence information has been achieved. Studying the genome sequence information of various pathogens to find out the genes conserved among the bacteria enables the identification of potential targets for the development of broad-spectrum antibiotics, while unique genes specific to particular species of bacteria might be an ideal target for narrow-spectrum antibiotics. For example, 26 genes in *E. coli* out of which most of them were conserved in various species such as *B. subtilis*, *M. genitalium*, *H. influenzae*, *H. pylori*, *Streptococcus pneumoniae*, and *Borrelia burgdorferi* genomes were identified by Arigoni F and colleagues (Arigoni et al. 1998). To potentially select the target, it is crucial to compare the genome sequence of the pathogen and the eukaryote so that the bacterial target proteins that are conserved among the mammalian proteins could simply be avoided to reduce the chances of human toxicity (Tatusov et al. 1997). For example, a previous report indicated significant sequence similarity between the broadly conserved proteins (15 out of 26) across the bacterial species and that of *Saccharomyces cerevisiae* (Arigoni et al. 1998).

5.3.3 Pan-Genomics

This is an advanced future of comparative genomics which aims at understanding the content, organization, and evolution of genomes and explains genotype-phenotype relationships. Availability of multiple genome sequences for a single species highlighted the importance of pan-genomics approach in identifying vaccine candidates in antigenically diverse species (Muzzi et al. 2007). The analysis of variation in genome sequences of pathogenic and its nonpathogenic strain leads to the rapid identification of genes involved in virulence. Pan-genomics focused on the variation in genomic sequence of different strains of same species which indicates that single genome sequence may not be enough or may not provide the complete understanding of intraspecies genetic variability (Fitzgerald et al. 2001; Dorrell et al. 2001; Fukiya et al. 2004; Obert et al. 2006). In pan-genomics approach, open reading frames (ORFs) are selected by screening of multiple genomes either by comparative genomics hybridization or by direct sequencing (Muzzi et al. 2007). These studies suggest that a potential vaccine and antimicrobial targets have to be conserved across all strains of the same species and are involved in the pathogenesis of bacterial pathogens. One of the best examples of genetic diversity studied through pan-genomics approach is seen in *Streptococcus agalactiae* (also known as group B streptococcus), a multiserotype bacterial pathogen that causes life-threatening disease in newborns. Genome sequence analysis of eight different strains of *S. agalactiae* predicted genetic variability and the extended collection of genes of the species (Tettelin et al. 2005). It can be classified into three parts: genes that are present only in one strain (strain specific genes), genes present in some strains but not in all strains (dispensable genome), and set of genes that are present in all strains (core genome). The bioinformatics screening predicted 589 genes as surface-exposed or

secreted proteins in the *S. agalactiae* genome. Among them, 396 and 193 genes are from the core and dispensable genome, respectively. Further, screening of these genes revealed four proteins that elicited protection in mice against all strains of *S. agalactiae* (Maione et al., 2005). Interestingly, it was found that a combination of four proteins GBS322, GBS104, GBS67, and GBS80 can act as a universal vaccine. However, only one of these proteins belonged to the core genome, while the rest of the three are from the dispensable genome of *S. agalactiae.* Therefore, the authors suggested that it is not the only conserved protein which essentially provide broad-spectrum protection (Kaushik and Sehgal 2008).

5.3.4 Transcriptomics

This genomics approach can be used for analysis of global changes in bacterial gene expression under a specific condition. Thus, genes which are essential for survival and pathogenesis of microorganisms in the host can be identified by the transcriptomic approach. The highly expressed genes can be selected for further analyses as they are crucial for microbial pathogenesis. On the contrary, low-expressed genes in host environment should be considered less important for a potential target. It is reported that targeting such genes which are shown to be essential for survival and expressed in virulence-induced condition has a higher potentiality to be drug target (Moir et al. 1999). Information about such essential genes that are also expressed in the animal model would indicate the importance of such genes in infection as well. There are commonly two types of methods for gene expression: first, cDNA-based microarray (cDNA derived from the RNA transcripts by using reverse transcription under specific condition) and second, ultra-high-throughput sequencing technologies that allow rapid sequencing and direct quantification of cDNA.

Identification of potential vaccine and therapeutic targets under experimental conditions by mimicking host-pathogen interaction is a good way. For example, in a study using microarray-based transcriptional profiling, it was found that adhesion to epithelial cells altered the expression of 350 genes by more than twofold, in which 189 genes were upregulated, 151 downregulated, and 7 genes either up- or downregulated depending on the time point in kinetics (Grifantini et al. 2002). They identified five new adhesion-induced proteins (NMB0315, NMB1119, NMB0995, NMB0652, and NMB1876) capable of inducing bactericidal antibodies in mice (Grifantini et al. 2002). However, there are some major limitations of this approach. Firstly, there was no direct correlation between the levels of proteins and mRNA. Secondly, in vivo studies require large amounts of mRNA; amplification of mRNA further creates additional technical challenges. Thirdly, they failed to establish a correlation between animal or cell-culture systems and the human host. Some other examples of microarray-based transcriptional profiling are (i) *Mycobacterium tuberculosis* genes encoding proteins that could be targeted for vaccine development, which are expressed during host infection (Talaat and Stemke-Hale 2005), and (ii) transcriptional profiling of *Vibrio cholerae* genes that are expressed during human infection (Merrell et al. 2002).

In addition to these techniques, several alternative techniques (in vitro expression technology (IVET), in vivo induced antigen technology (IVAT), and expression library immunization) are also developed for the study of bacterial gene expression globally (Angelichio and Camilli 2002; Talaat and Stemke-Hale 2005). Besides these techniques, signature-tagged mutagenesis (STM), genome analysis and mapping by in vitro transposition, and transposon site hybridization (TraSH) techniques are also developed with the special emphasis on the bacterial genes whose expression is dependent on host-pathogen interaction. The idea behind the development of such high-throughput techniques is to find out number of vaccine and therapeutic targets from bacterial species (Merrell et al., 2002; Moxon and Rappuoli 2002; Scarselli et al. 2005).

5.3.5 Proteomics

Proteomics refers to analyzing a set of proteins expressed under specified conditions or in specific cellular location. Using this approach, the potential vaccine and therapeutic targets could be predicted by obtaining an overall view of the pathogen proteome and the host's immune response after infection. High-throughput proteomic analysis can also be performed by using several techniques such as mass spectrometry, chromatographic techniques, and protein microarrays (Grandi 2006). One of the chromatographic techniques like 2D-PAGE separates proteins that appear as fine spot on the gels; these are then isolated and subjected to further analysis by mass spectrometry. Mass spectrometric techniques such as MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) and MS/MS (tandem mass spectrometry) are used for peptide mass and sequence analysis of protein spots on a gel (Patterson and Aebersold 2003; Zhu et al. 2003). One of the common examples of proteomics-based approach is the identification of 27 outer surface proteins of *S. agalactiae*, first by 2D-gel electrophoresis and then by peptide sequencing. Out of these, six proteins were cloned, expressed, purified, and then utilized for mice immunization experiments. Two potential candidates were found to be protective against a lethal dose of bacteria in a neonatal mouse model (Hughes et al. 2002). Grandi (2006) also analyzed the surface proteome of *Streptococcus pyogenes* to identify novel vaccine and therapeutic targets (Rodriguez-Ortega et al. 2006). This novel proteome-based approach is used to identify novel proteins in several organisms such as *Bacillus anthracis* (Ariel et al., 2003), *Streptococcus pneumoniae* (Ling et al., 2004), *Streptococcus iniae* (Shin et al., 2007), *Bartonella quintana* (Boonjakuakul et al., 2007), and *Mycobacterium tuberculosis* (Malen et al., 2008).

5.3.6 Immunomics

Immunomics is the analysis of a set of proteins and epitopes of the pathogen that interact with the host immune system. The proteome of bacteria can also be screened

to identify immunome of that bacterium by in silico and in vitro techniques. In silico techniques can be used to predict pathogen epitopes that can be recognized by B-cell and T-cell. Large-scale screening for B-cell and T-cell epitopes in pathogens including HIV, *B. anthracis*, *M. tuberculosis*, *F. tularensis*, *Yersinia pestis*, flaviviruses, and influenza is currently under process (Sette et al. 2005; De Groot et al. 2008a). Although epitope prediction may serve as a steer for further biological evaluation, T-cell epitopes are recognized by MHC/HLA complex on the surface of antigen-presenting cells (B-cell, macrophages, and dendritic cells), which differ considerably between hosts, confounding the task of functional epitope prediction. Furthermore, B-cell epitopes can be both linear and conformational. Finally, the rationale behind the study was to create a single peptide which could represent defined epitope combinations from a protein or organism and overcome the genetic variability of both pathogen and host (De Groot et al. 2008b).

Antibodies present in host serum upon exposure to a pathogen can be used to identify vaccine candidates. There are several established techniques which allow the high-throughput display of pathogen proteins and the subsequent screening for proteins that interact with antibodies present in host serum (Seib et al. 2009). Immunogenic surface proteins of various organisms have been identified in several studies, including *Staphylococcus aureus* using 2D-PAGE, membrane blotting, and MS (Vytvytska et al. 2002); *Streptococcus agalactiae*, *Streptococcus pyogenes*, and *S. pneumoniae* using phage- or *E. coli*-based comprehensive genomic peptide expression libraries (Meinke et al. 2005; Giefing et al. 2008); and *Francisella tularensis* (Eyles et al., 2007) and *Vibrio cholerae* using protein microarray chips (Rolfs et al. 2008). Characterization of protein-drug interactions, as well as other protein-protein, protein-nucleic acid, ligand-receptor, and enzyme-substrate interactions, can also be done by using protein microarray (Stoevesandt et al. 2009).

5.3.7 Structural Genomics

Structural genomics mainly focuses on the three-dimensional structure of an organism's proteins and how they interact with antibodies and therapeutics. NMR (nuclear magnetic resonance) and crystallography techniques are used to determine the structure of proteins and the conformational changes that occur during the interaction of proteins with antibodies and therapeutics. This approach is quite useful to engineer antibodies and inhibitors against specific proteins by using their structure-based design to find out the residues involved in the active site of that protein. High-resolution techniques for protein structure determination are mainly focused on understanding and analyzing the structural basis of immune-dominant and recessive antigens as well as active sites and potential drug binding sites of proteins (Dormitzer et al. 2008; Nicola and Abagyan 2009). Several methods have been developed for high-throughput characterization of proteins on the basis of their genome information (Todd et al. 2005). For example, structural characterization of two HIV

envelope proteins gp120 (glycoprotein 120) and gp41 (glycoprotein41) have shown mechanisms used by the virus to evade host antibody responses due to hypervariability in immunodominant epitopes (Zhou et al. 2007; Prabakaran et al. 2007). However, there are some limitations to this approach such as poor understanding of determinants of immunogenicity, immunodominance, and structure-function (Seib et al. 2009). Nevertheless, this approach is very important for high-throughput modification of proteins and their screening for immunogenicity and interaction with antimicrobials to develop some novel vaccine and therapeutics (Dormitzer et al. 2008).

5.4 Conclusions

In this chapter, we review the impact of microbial genomics approach on the development of novel vaccine and therapeutics. This chapter covers several microbial genomics approaches that have emerged to identify the potential candidates for vaccine and therapeutic design from our better understanding of the human genome. Genomic and proteomic approaches have been used to identify the surface proteins during host-pathogen interaction. Furthermore, transcriptomics tells us about the expression level of RNA transcripts during infection, which is useful to dig out the essential target for the development of vaccine and antibiotic targets. All these approaches are useful, but there still remain some challenges such as understanding of molecular nature of B-cell and T-cell antigenic determinants of immunogenicity, mechanisms of different adjuvants, and structure-function relationship of proteins. These challenges can be fulfilled by improvement of structural studies of antigenic determinants, immunogenicity, and B-cell and T-cell epitope prediction. Identification of novel vaccine and therapeutic targets through genome-based approaches has to be subjected to confirmation and validation by in vitro (e.g., bactericidal assay) and in vivo assays (e.g., animal protection experiments). Unavailability of valid models to measure efficacy and protection against disease is still a major issue of animal protection experiments. In spite of that, a wealth of information about the microbial pathogenesis obtained through genome-based approaches can be useful in sorting out this issue. Several effective vaccines and therapeutic candidates have to pass through confirmatory tests including stepwise series of pre-licensure clinical trials (phases I, II, and III) before being introduced into the market. However, preclinical trials that are required to check the safety, efficacy, and immunogenicity of potential vaccine and therapeutic targets are time-consuming and costly. We therefore believe that with advancements in the field of technology, we can expect to witness more effective and specific vaccine and therapeutic targets against a disease in the near future.

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6 Next-Generation Sequencing (NGS) Platforms: An Exciting Era of Genome Sequence Analysis

B. Meera Krishna, Munawwar Ali Khan, and Shams Tabrez Khan

Abstract

DNA referred to as blueprint of life codes for the diversity and function of all the living organisms. Determining DNA sequences of the living organisms not only gives an overview of their genetic makeup, but also provides information about their function. Nonetheless it was not easy to determine the genome sequencing of all the diversity around us especially with the technologies available before 2010. Therefore, determining the sequence of humans and some other organisms only was prioritized. Pioneering methods for DNA sequencing given discovered by Maxam and Gilbert, and Sanger although were very powerful and popular but were not high throughput and economic. Therefore, it was necessary to develop new economic and high-throughput methods that can sequence the biodiversity consequently providing better insights of their possible function. New methods were developed and commercialized by Roche Life Sciences, Thermo Fisher Scientific, Illumina, and Applied Biosystems. These methods generally referred to as next-generation sequencing methods have revolutionized the DNA sequencing. Many sequencing platforms employing NGS have been developed including pyrosequencing, Ion Torrent technology, Illumina/Solexa platform, and SOLiD (Sequencing by Oligonucleotide Ligation and Detection). Further optimization has led to innovative third and fourth-generation platforms as single molecule real-time (SMRT) sequencing by PacBio, nanopore sequencing, etc. As a consequence there is a sharp increase in the number of genomes being published and other genome-based studies since 2012. This has made it easy even to imagine of

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sequencing the genomes of individuals. Furthermore, scientists are now looking for third-generation sequencers that may be significantly different from the sequencers that are currently available.

Keywords
Genome · Next-generation sequencing (NGS) · NGS platforms · NGS chemistry · NGS applications

6.1 Introduction

Since the discovery of DNA as the genetic material by Frederick Griffith in 1928, mankind continues its advance in improving the DNA-based technology and to unravel the blueprint of life (Avery et al. 1944). One of the most important progresses that has been made in understanding the blueprint of life is the improvements made in sequencing technologies. The initial methods developed by Maxam and Gilbert were able to sequence only a few nucleotides, which was followed by the development of chain termination methods of Sanger. It was however realized during the Human Genome Project in 2003 (National Human Genome Research Institute (NHGRI), NIH 2003) that high-throughput and economic methods are required to complete the HGP and other future projects. Later on next-generation sequencing methods were developed that made sequencing high throughput and generated the data faster than ever. It is interesting to note that since the development of these sequencing techniques, genomes of a number of organisms have been published. These approaches have provided better insights of the complex microbial environments such as gut microbiome, etc. Scientists are still working further to make these methods economic and high throughput.

6.1.1 DNA Sequencing: History

For years, a variety of technologies and tools have been used for genome sequence analysis. DNA sequencing can be defined as the process of determining the sequence of nucleotides (As, Ts, Cs, and Gs) along the length of a DNA molecule. The very first method to sequence the DNA was Maxam-Gilbert method. However, this method was only able to sequence a few nucleotides at a time. This was followed by the discovery of chain termination method which was developed by the British biochemist Frederick Sanger and his colleagues in 1977. For the development of this powerful technique, Sanger and his colleagues were awarded the Nobel Prize in chemistry in1980 (Sanger and Coulson 1975). Sanger sequencing was very powerful since it was able to sequence about 900 base pairs at a time compared to a few bases that can be sequenced using Maxam-Gilbert method (Sanger et al. 1977). Even with the development of Sanger sequencing technology, it was a momentous task to correctly sequence 3 billion base pairs of human genome. Therefore,

sequencing using Sanger's method is expensive and time taking (Ari and Arıkan 2016). Past decade has witnessed a revolutionary progress in developing high-throughput sequencing significantly contributing to genomics. These technologies have become high throughput mainly due to the improvement in new and efficient detection tools and miniaturization of the available technologies. Automated DNA sequencers like those developed by PE Biosystems and GE Healthcare have also been witnessed in the last two decades. Next-generation sequencing (NGS) is now synonymous to high-throughput sequencing, producing and analyzing millions of sequences per run. This has made sequencing, resequence, and comparing the data much faster. This has enabled extensive analysis of living systems and their genomics (Nowrousian 2010).

6.1.2 Next-Generation Sequencing

Sanger method was used mainly as the only sequencing method for three decades, despite its high cost and time as major drawbacks. Next-generation sequencing (NGS) technologies are emerging as one of the most economic, quick, and high-throughput methods of DNA sequencing. A number of different platforms based on these technologies are being currently used for sequencing, such as:

- Roche/454 sequencing
- Ion torrent: Proton/PGM sequencing
- Illumina (Solexa) sequencing
- SOLiD sequencing

Where Sanger technique was considered as the first-generation method, such plateforms are recognized as the second-generation tool. The technique was firstly reported in 2005 by Roche's 454 technology and was commercialized as technology capable of generating high-throughput sequence data, at much lower cost than the first-generation sequencing technologies (Qiang-long et al. 2014). NGS offers many benefits in comparison with the traditional sequencing methods as well as the microarray expression profiling. The basic advantages of NGS technology are (1) high throughput, the generation of multiple short reads in parallel, (2) fast, (3) economic, (4) wide range of detection, and (5) discreteness (the results are generated without noise and signal saturation). The advantages of NGS over Sanger method and microarray is summarized in Table 6.1.

The sequence data produced by the second-generation sequencing comprises of billions of short DNA sequences (reads) that range from 50 to 300 nt in length. These sequences require *de novo* assembly before the analysis.

Short-read sequencing methods are divided under two wide categories: (1) sequencing by ligation (SBL) and (2) sequencing by synthesis (SBS) (Goodwin et al. 2016; Myllykangas et al. 2012). Sequencing by ligation (SBL) exploits the mismatch sensitivity of DNA ligase to fix the underlying sequence of nucleotides in a given DNA sequence. Sequencing by synthesis (SBS) utilizes DNA polymerase or

Table 6.1 Comparison of NGS technology with Sanger sequencing and microarray in terms of its advantages

NGS vs Sanger sequencing	NGS vs microarray
1. High sensitivity: higher sequencing depth enables higher sensitivity (down to 1%)	1. Discovery mode: detection of novel transcripts, gene fusions, single nucleotide variants, indels (small insertions and deletions), and other previously unknown changes without the use of probes
2. Higher discovery power: identification of novel variants by increasing the number of targets sequenced in a single run	2. Broad dynamic coverage: NGS outcomes are discrete, digital sequencing read counts, and can quantify expression across a larger dynamic range. The results are not limited by background at the low end and signal saturation at the high end
3. Higher mutation resolution	3. Higher specificity and sensitivity: NGS allows detection of a higher percentage of differentially expressed genes and also genes with low copy numbers
4. Higher sample throughput	4. Simple detection of rare and low-abundance transcripts: the broad range of sequence coverage also allows detection of rare transcripts, single transcripts per cell, or weakly expressed genes
5. Higher detection limits: sequencing samples that have low input amounts starting from 10 ng of input DNA and enormous data produced with the same amount of input DNA compared to Sanger method	
6. Sequencing of emergency situations as outbreaks	

ligase enzymes to encompass many DNA strands concurrently. Nucleotides or short oligonucleotides are introduced either on a single time or modified with identifying tags so that the base type of the incorporated nucleotide or oligonucleotide can be recognized as the extension happens. Figure 6.1 explains the mechanism behind the sequencing by synthesis (SBS) and sequencing by ligation (SBL) briefly. Different chemistry used by three popular NGS platforms (Roche/454 sequencing, Illumina/Solexa sequencing, and ABI/SOLiD sequencing) is discussed below briefly.

6.1.2.1 Roche/454 Sequencing

Roche/454 sequencing is the pioneer NGS platform and was launched in 2005 by Rothberg and colleagues and is popularly referred to as pyrosequencing (Margulies et al. 2005). The technique is based on sequencing-by-synthesis approach and uses pyrosequencing with pyrophosphate (PPi). The technique was developed by Ronaghi et al. (1996). The chemistry of pyrosequencing is based on the detection of pyrophosphate which is released during the incorporation of a nucleotide in the newly synthesized DNA strand. Sequencing primer is hybridized to a single-stranded biotin-labeled template and mixed with the four enzymes: DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates adenosine 5′ phosphosulfate (APS) and luciferin (Gharizadeh et al. 2007; Nyren and Skarpnack 2001). For sequencing, DNA samples are randomly fragmented, or amplicons of

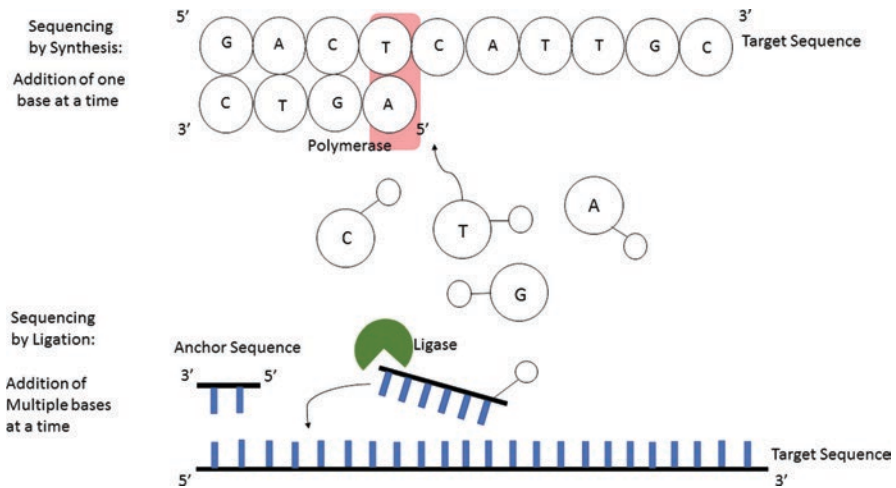

Fig. 6.1 The chemistry behind the sequencing by synthesis (SBS) and sequencing by ligation (SBL)

suitable sizes are generated. In the next step of library preparation, the fragments are ligated to 454 sequencing adaptors. The adaptor enables the attachment of DNA fragments to the streptavidin beads whose surface carries primers that have oligonucleotides complementary to the DNA fragments. Each bead is associated with a single fragment and is captured into individual emulsion droplets. The reaction cascade starts with the addition of deoxynucleotide triphosphates (dNTPs) to the PCR mixture. These droplets are subjected to emulsion PCR (emPCR) creating millions of copies of each DNA fragment on the surface of each bead. The output from one bead corresponds to one read. The template beads are then transferred to picotiter plate (PTP), a surface containing nanometer size wells for pyrosequencing. The sequencing reaction progresses through a series of downstream reactions producing light upon the incorporation of nucleotide. Each DNA fragment will be synthesized in one well using a pyrosequencing reaction. The utility of PTP makes possible the run of thousands of pyrosequencing reactions in parallel, increasing the sequencing throughput to a higher extent (Mardis 2008). The specific nucleotide added is detected by quantifying the light emitted with the conversion of PPi to ATP after the incorporation of each nucleotide by DNA polymerase (Mardis 2008). In each cycle a CCD camera captures signals from spots on PTP corresponding to the position of unique DNA fragments. The recorded images are processed for base calling a process entailing quantitative correlation of light signals with specific order of added nucleotides and decide nucleotide content of every DNA fragment.

The Roche/454 sequencing can generate reads with lengths of up to 1000 bp for genomic DNA and up to 600 bp for amplicons. It can produce ~1Million reads per run. Typical throughput of Roche systems ranges from 450 to 700 Mb. The Roche/454 is able to generate relatively longer reads which made it further easier to map the genomes. The consensus accuracy of Roche's FLX+ platform is reported to

be 99.9997% at 15X coverage for *E. coli* genome. The method allows real-time detection of the base incorporation as it requires no electrophoresis to separate the extension products (Fakruddin et al. 2012). The method was used for sequencing of difficult secondary structures like hairpin (Ronaghi et al. 1999) and inverted terminal repeats (ITR) of adenoviruses (Petri et al. 2014). The main drawback of the pyrosequencing platform is the high error rate in accurately sequencing homopolymer regions and regions characterized by insertions and deletions (Huse et al. 2007; Margulies et al. 2005). These errors in the accurate identification of nucleotides arise due to the misinterpretation of the number of nucleotides wherever many similar nucleotides are being repeated resulting in very high signal intensity. The second major disadvantage of the method is the prolonged sample preparation protocols based on emPCR. The small length of the reads obtained through pyrosequencing technology compared to those obtained through Sanger sequencing technology is also a disadvantage of the technology. As the sequence length is crucial for effective assembly, long reads may minimize the problems associated with large genome size, high-repetitive DNA ratio, and various ploidy levels (Schatz et al. 2012). Roche Diagnostics Corporation announced shutting down 454 updates in 2013 and gradually finished the production of reagents of 454 platforms.

Ion Torrent: Proton/Personal Genome Machine (PGM) Sequencing

Another sequencing platform for next-generation sequencing is Ion Torrent semiconductor sequencing technology commercialized by Thermo Fisher. This technique was first developed by Toumazou et al. in 2006 and is generally referred to as sequencing-by-synthesis (Rothberg et al. 2011). Here the addition of nucleotides is detected through the generation and detection of an electrochemical signal more specifically through the generation and detection of a hydrogen ion. The sequencing reaction takes place on a complementary metal-oxide semiconductor (CMOS) chip referred to as flow cell. But instead of using fluorescently labeled nucleotides or chemiluminescence like other next-generation sequencing technologies, it directly measures the emission of H+ ions during the incorporation of dNTP to a growing DNA strand (Rothberg et al. 2011). The release of H+ ions during the process changes the pH in the microenvironment by forming a high positive voltage. This change of current can be detected by a transistor-based device which is eventually converted into a voltage signal (Merriman and Rothberg 2012).

The chip used for sequencing contains set of micro wells, and each well has a bead with several identical DNA fragments. An ion-sensitive (ISFET) fashion sensor deployed to the bottom of a microwell acts as pHFET (pH-sensitive field effect transistor). The sequencing starts with the fragmentation of DNA or RNA into pieces of uniform size of approximately 200–400 bp. The carrier bead typically carries large numbers of clonally amplified population of short DNA templates, formed by emulsion PCR (emPCR) process. The DNA hooked to a single bead in different well serves as template for sequencing reaction. The well retains the beads in place and also confines the H^+ ions released during the addition of each nucleotide. As described above the corresponding pH change will be detected by the ISFET and

converted into a voltage signal proportionate to the number of nucleotides incorporated.

The Ion Torrent sequencers generate rather longer reads lengths of 200 bp, 400 bp, and 600 bp compared to other NGS platforms. What makes Ion Torrent platform unique is that it uses unmodified nucleotides. So instead of fluorescently labeled dNTPs, Iron Torrent technology uses unmodified bases and the signals are also not detected as specific flourescence, but the signals are generated as H+ ions. The approach errands better enzyme activity, long reads, and economic consumables. In the absence of low image scans, "base calling" processes are accomplished at a much faster pace. According to the recent standards, Ion Torrent platforms can reach up to 10 Gb sequencing data per run at a time span of 2.5 h (Ari and Arıkan 2016). The major disadvantage is the difficulty of interpreting the homopolymer sequences (more than 6 bp) inducing higher error in specific regions (Loman et al. 2012; Morey et al. 2013; Reuter et al. 2015). The sequence chemistry doesn't allow the reaction to pause after each base incorporation; bits of the same base will effect in a single, albeit sturdier signal. This feature makes identification of longer sequence stretches with similar bases difficult.

Illumina/Solexa Sequencing

In the mid-1990s, scientists at Cambridge, Shankar Balasubramanian and David Klenerman, developed methods to produce high-quality reads of much greater data size at a reduced cost. In this method single DNA molecules are attached to a flat surface, amplified *in situ*, and sequenced using fluorescent reversible terminator deoxyribonucleotides. The florescent signals generated during the reaction are recorded as images. Finally, the images of the surface are analyzed and processed to generate high-quality sequence data (Bentley et al. 2008). Researchers later on founded Solexa company which was later acquired by Illumina in 2008, and the technology was then referred to as Illumina sequencing technology (http://www.illumina.com). These were further commercialized by Solexa as Ilumina/Solexa Genome Analyzer (GA) (Balasubramanian 2015; Shendure and Ji 2008). Presently, the company owns MiSeq, NextSeq 500, and HiSeq 2500 platforms that produce 15 Gb, 120 Gb, and 1000 Gb of sequencing data per run and have maximum 2 × 300 bp, 2 × 150 bp, and 2 × 125 bp read length, respectively.

Illumina sequencing is currently the most popular technology in the NGS market and is responsible for more than 90% of the world's sequencing data generated (Illumina 2017). Illumina method uses sequencing-by-synthesis chemistry joining bridge amplification on a solid surface (Adessi et al. 2000) developed by Manteia Predictive Medicine and reverses termination chemistry and engineered polymerases (Bennett 2004) established by Solexa.

The general mechanism of Illumina sequencing has four basic steps: library preparation, cluster generation, sequencing, and data analysis. During the library preparation step, the DNA or cDNA samples are randomly fragmented into sequences followed by 5′ and 3′ adapter ligation and index sequences. The adaptor-ligated fragments are PCR amplified and gel purified. For cluster generation, the library of adaptor-ligated fragments is loaded onto a flow cell where these

fragments are hooked on a slide of surface-bound oligos complementary to them. Each attached adaptor fragment is then amplified by "PCR bridge amplification" into several distinct copies, each representing the same original sequence called as the "clonal clusters." The cluster generation enables production of sufficient signal during imaging process. Next the templates are sequenced by the technique called as sequencing by synthesis based on reversible terminator method that detects single bases as they are incorporated into DNA template strands. Here DNA polymerase adds one of four different fluorescently modified nucleotides to a growing DNA chain (Bentley et al. 2008). The modified nucleotides also contain an inactive 3′ hydroxyl group referred as the blocking group to ensure that only one nucleotide is incorporated growing DNA chain. Clusters are excited by laser for emitting a characteristic light signal specific to each nucleotide incorporated. The optic signals are detected by a coupled-charge device (CCD) camera, and computer programs translate these signals into a nucleotide sequence. Subsequently, 3′ blocking group and fluorescent dye are removed from nucleotide structure to enable the addition of nucleotides in the next cycles. The number of cycles determines the length of the read. The emission wavelength along with the signal intensity governs the base calling. For a given cluster, all identical strands are read simultaneously, and hundreds of clusters are sequenced in a massively parallel process. The entire process generates millions of reads representing all the fragments (Reuter et al. 2015; Heo 2015). For data analysis sequences from the sample libraries are separated based on the unique indices presented during the library preparation. For unique samples reads with analogous strings of base calls are locally clustered, forward and reverse reads are matched to result in contiguous sequences. These contiguous sequences are aligned to the reference genome for species/variant identification (www.illumina.com/SBSvideo).

The pioneer sequencers Illumina/Solexa GA have been capable of making very short reads ~35 bp and had a selective advantage of producing paired-end (PE) short reads, with the sequences at both ends of each DNA cluster is documented. Further refinements and optimization led to the manufacturing of the latest generation of Illumina SBS technology-based instruments which can generate multiple terabases (Tb) of data per run. The latest Illumina sequencers produces an output data greater than 600 Gb and short read length of about 125 bp. Illumina platforms are reported to have 99.9% accuracy, and with standard reagents, barcoding of 96 samples per run can be performed (Morey et al. 2013). Illumina sequencing technology has its own advantages and disadvantages. The library preparation time less than 90 min is compared to earlier platforms (Illumina 2014). It has significantly improved the high-throughput data while reducing the cost and time for each run (Buermans and Den Dunnen 2014). The error rates of Illumina method are very low attributed to the increased competition of all four reversible terminator-bound dNTPs present during each sequencing cycle. The highly accurate base-by-base sequencing is made possible through the use of blocking groups which also eliminates the possibility of errors, even in homopolymer regions (Ross et al. 2013; Bentley et al. 2008). Therefore, Illumina sequencing platforms are better for sequencing homopolymeric regions than other platforms (Mardis 2013). Irrespective of its superior

performance, the Illumina/Solexa platform also has some limitations. One of the major problems with Illumina/Solexa platform is sample loading control as overloading may result in overlapping clusters leading to poor sequencing quality. The error rate of the sequencing technology is about 1%, and substitution errors of nucleotides are the most frequent type of error (Dohm et al. 2008; Hutchison 2007). The efficiency of the sequencing reactions can be reduced due to the contamination of proteins and the altered nucleotide structure resulting from the errors in cleavage of blocking group (Chen et al. 2013). The bridge amplification is also sensitive to GC content variation of the DNA. It is also evident from the fact that GC-rich regions of heterogenous genomes are underrepresented in sequences obtained using Illumina method (Tilak et al. 2018). The major error in the Illumina sequencing is known as phasing. Briefly, phasing occurs when the blocker of a nucleotide is not properly removed after signal detection. It will block the binding of new nucleotide onto the DNA fragment in the next cycle, and the old nucleotide is detected again, whereby the fluorescence signal of this old nucleotide differs from the synchronous signal of the other nucleotides. This miscorporated DNA fragment will be one cycle behind the rest (out of phase), generating asynchronous light signals that get read by the camera. Since the signal intensity is the measure to calculate the quality scores, the "out of sync" signal results in a decreasing sequence quality score. This creates the major flaw in the Illumina sequencing, i.e., read length limitation and compromised quality. This presents perceptible hurdles in various applications especially in *de novo* sequencing (Chen et al. 2013).

ABI/SOLiD Sequencing

Supported Oligonucleotide Ligation and Detection (SOLiD) is another NGS platform marketed by Life Technologies (http://www.lifetechnologies.com). In 2007, Applied Biosystems (ABI) has acquired SOLiD, and the first ABI/SOLiD sequencing system was commercialized. The SOLiD sequencing works on the basis of sequencing by ligation (SBL) approach (Shendure and Ji 2008). Sequencing by ligation uses the mismatch sensitivity of DNA ligase to determine the nucleotide sequence (Ho et al. 2011). The basic concept was first demonstrated by Jay Shendure and grouped to resequence an evolved strain of *Escherichia coli* at a lesser error rate of 10^{-6}. The technology employs a cell-free, mate-paired library construction of DNA fragments, bead-based emulsion PCR of the molecules to construct "polonies" hence also known as polony "sequencing," immobilization of the beads onto a polyacrylamide gel-based microscope glass and automated sequencing by ligation, and fluorescent four-color imaging specific to labels of nucleotide sequences (Shendure et al. 2005). In principle, the chemistry exploits two-base encoding, a di-base (two-base) query system for probing the sequence and a fluorescent dye for detection. The system uses 4 fluorescent dyes to interrogate all 16 (4^2) possible two-base combinations and several modified 8-mer probes. The first two bases at the 5′-end of the probes characterize the exclusive two-base combination, and the 3′-end is tagged with the fluorophore (Choudhuri 2014). The rest of the octamer is degenerated in nature which can bind to any DNA sequences.

The ABI/SOLiD process has four main steps: library preparation, emulsion PCR/bead enrichment, bead deposition, and sequencing by ligation/data analysis. Based on the purpose, two types of libraries are generated: fragment or mate-paired. Clonal bead population or polonies are generated by emulsion PCR over microreactors containing template, adaptors, PCR reaction components, beads, and primers. The product templates are denatured and the undesired beads with extended templates separated through bead enrichment. The template on the selected beads undergoes a 3′ modification to allow covalent bonding for the later immobilization step. Next 3′ modified beads are deposited onto a glass slide. While the beads are loaded, the deposition chambers can segment a slide into one, four, or eight chambers. This feature enables the accommodation of larger input densities of beads per slide, contributing to the high throughput of the system. For the sequencing the primers are hybridized to the P1 adapter sequence within the library template. A set of four fluorescently labeled di-base probes compete for ligation to the sequencing primer for extension of the templates. Cycles of ligation, detection, and cleavage are repeated in multiple numbers defining the ultimate read length. After each step of ligation, the extension product is washed, and the template is primed with the complementary primer to the n-1 position for the next cycle. The color emitted will be captured as the signal and analyzed. The recovered data can be translated to deduce the sequence of the DNA fragment in question (Applied Biosystems 2008).

ABI/SOLiD first sequencer generated short reads with length 35 bp and an output of 3 Gb/run. Later process optimization improved the paired read length to 2X50 with an output up to 320 Gb/run (Alic et al. 2016; Goodwin et al. 2016). A sequencing run generally takes 7–14 days to finish the task. The latest commercial SOLiD platforms (5500 W and 5500xl W) have 99.99% accuracy. The main strengths of the ABI/SOLiD platform are its unrivaled accuracy inherent due to unique chemistry and analysis features: high fidelity ligation reactions, two-base encoding technology, primer resets, and mate-paired libraries. The SOLiD workflow and system are robust allowing real-time run tracking and workflow reentry without duplicating the sample or slide. The system flexibility is another remarkable feature of the ABI/SOLiD platform. The users can perform two completely independent experiments on a single SOLiD analyzer conferred by independent flow cell configuration. Analysis of sequencing, expression profiling, or sequencing of multiple mate-paired libraries is made possible with the usage of different insert sizes in a single run. Sample multiplexing feature also increased the flexibility of the system and decreased the cost and time requirements (Applied Biosystems 2008). These features are used for detection of copy number variations (CNV), single nucleotide polymorphisms (SNPs) and microbial phylogeny analysis but are well suited for resequencing projects requiring low error rates and transcriptome sequencing (Metzker 2010).

The main type of error in this platform is also substitution. The provenance of the errors is the noise during the ligation cycle leading to the misinterpretation of the bases. The quality of the reaction can be compromised due to the fluorescent leakage and cross talk between high density DNA template beads and impromptu mixing of beads with DNA fragments. This can cause reduction in filtered data sets up

Table 6.2 Comparison of the main features of the popular next-generation sequencing platforms

Platform	Maximum throughput Mb/run	Mean length (nucleotide)	Error rate	Specific features	Origin of errors
Roche/454	700	Up to 1000 bp	10^{-3}–10^{-4}	Long read lengths (improved mapping in repetitive regions, short runtime	Homopolymers, intensity cutoff, signal cross-talk interference among neighbors, amplification, mixed beads
Ion Torrent PGM	1000	~200	3×10^{-2}	Stable sequence quality, better sequencing GC depth distribution	Homopolymers, amplification
Illumina	6000	~100	10^{-2}–10^{-3}	Highest throughput Long-/short-run times, low capital cost, low-cost per Mb	Signal interference among Neighboring clusters, homopolymers, phasing, nucleotide labeling, amplification, low coverage of AT rich regions
SOLiD	20,000	~50	10^{-2}–10^{-3}	High throughput, highest accuracy two-base encoding provides inherent error correction	Intensity cutoff, homopolymers, signal cross-talk interference among neighbors, amplification, mixed beads

to 50% (Pickrell et al. 2012). False reading and low-quality bases can be introduced by the short distance between the beads. The error rates can multiply as the ligation cycles proceeds, if signal intensity is diminishing due to improper removal of fluorescent dye (Ari and Arıkan 2016). Incompetent data analysis and hindrances in sequencing of palindromic sequences are other concerns to be addressed in the ABI/SOLiD platform (Bao et al. 2011; Huang et al. 2012). Table 6.2 comprises the main features of each platform.

6.1.2.2 Applications

NGS platforms permit an extensive range of methods, allowing researchers to address questions related to genome, transcriptome, or epigenome effectively. The breadth of these applications makes the platforms ideal choice for research, clinical diagnosis, agriculture, and sustainable development. We are briefing the main applications achieved through the NGS platforms in the following section.

Resequencing

Resequencing is the most relevant application of NGS platforms. Through aligning with a reference sample, it enables the identification of genomic variations in the sample of our interest. This exploits copy number variation (CNV) analysis (Marmontel et al. 2018; Zimmerman et al. 2018), gene mutation analysis (Hsiao et al. 2016; Huang et al. 2017), genotyping (Segawa et al. 2017; Suzuki et al. 2018), and single nucleotide polymorphism (SNP) analysis (Ahmadian et al. 2000; Milan et al. 2000; Nordström et al. 2000; Marmontel et al. 2018; Zhang et al. 2019; Scaduto et al. 2010).

Whole Genome Sequencing

In whole genome sequencing, the difference between the DNA of specific whole genome is aligned to a reference genome and used to identify the conspicuous differences among the two. It encompasses a broad array of variations as large as possible (Cross et al. 2014; Hedges et al. 2011; Hodzic et al. 2017; Morozova and Marra 2008; Rosse et al. 2017; Shen et al. 2008; Shirasawa et al. 2013; Stothard et al. 2011). Whole genome sequencing using Illumina platform has produced significant output in sequencing agriculturally important livestock, plant genomes (Ichida and Abe 2019), or disease-related microbial genomes (Rohit et al. 2019).

Targeted Resequencing

Targeted resequencing is a type of resequencing that allows sequencing of a small part of genome like exome (Gorski et al. 2019; Nichols et al. 2012; Fujita et al. 2017), transcript (Strengman et al. 2019), or a particular gene of interest (Harismendy et al. 2009; Bhan et al. 2019; Elert-Dobkowska et al. 2019; Szelinger et al. 2011). This allows the users to concentrate on the low-level variations that can be missed while considering whole genome.

Transcriptome Sequencing

Transcriptome sequencing includes a variety of applications from transcriptome sequencing (Mangul et al. 2014), transcriptome profiling (Allie et al. 2014; Chi et al. 2012), discovery and identification of novel coding and noncoding RNAs such as miRNAs (Bhan et al. 2019; Johansen et al. 2011; Li et al. 2010; Hu et al. 2017; Veeranagouda et al. 2017), ribosome profiling (Abernathy and Overturf 2016; Kanda et al. 2015; Song et al. 2018), and identification of microsatellites/sequencing of amplicon, BAC, fosmid, etc. (Schatz et al. 2012). The techniques used for the purpose are RNA-seq, small RNA-seq, or tag-based approaches (Nourizad et al. 2003). Recent study has used this application for prediction of fetal blood group and platelet antigens from maternal plasma (Orzinska et al. 2019).

De Novo Sequencing

De novo sequencing generally refers to the sequencing and identification of novel genomes in the absence of a reference genome for alignment. Here sequence reads are congregated as longer contiguous sequences known as contigs or correctly arranged contigs known as scaffolds. This application is of particular interest in

comparative genome studies. Roche technology was considered ideal for *de novo* sequencing. This particular application has been widely used for clinical diagnosis (Minei et al. 2018; Cui et al. 2017).

ChIP Sequencing (Chromatin Immunoprecipitation Sequencing)

This application enables the identification and analysis of DNA protein interaction and sheds light on regulation of gene expression (Barozzi et al. 2011; Marklund and Carlborg 2010; Stothard et al. 2011; Prins et al. 2018; Rai and Adams 2016). It can determine the sequences interacting with particular transcription factor and overall chromatin architecture.

Microbial Sequencing

The NGS enables variety of microbial studies such as metagenomics to identify microbes in environmental samples, bacterial, viral and fungal typing, mutation analysis (Petronella et al. 2018), metabarcoding (Braukmann et al. 2019; Forin-Wiart et al. 2018), etc. Roche platform was widely in practice for bacterial (Gharizadeh 2003; Gharizadeh et al. 2005; Jonasson et al. 2002; Grahn et al. 2003) and fungal species (Trama et al. 2005a, b; Xia et al. 2016) phylogeny study and viral typing (Adelson et al. 2005; Elahi et al. 2003; Gharizadeh et al. 2001; Gharizadeh et al. 2003; Gooneratne et al. 2014; Tumiotto et al. 2017). But Ion Torrent technology has been considered as a perfect tool for 16sRNA sequencing (Salipante et al. 2014) for bacterial and fungal community profiling (Fujimoto et al. 2014; Sirichoat et al. 2018; Tremblay et al. 2019) and viral typing (Ari and Arıkan 2016).

Methylation Sequencing

NGS platforms permit to study the methylation state of DNA on a truly genome-wide approach to generate the "methylome." Currently, the methylome sequencing is achieved by either whole genome bisulfite sequencing (WGBS) or methylated DNA immunoprecipitation (MeDIP-Seq). Methylation sequencing allows us to study the methylation state of genome and how it regulates the gene expression. Multiple studies have successfully utilized methylation sequencing for revealing epigenetic control of gene expression (Rickert et al. 2002; Uhlmann et al. 2002; Neve et al. 2002; Gu et al. 2019; Harlid et al. 2019; Hearn et al. 2019; Niyomnaitham et al. 2019).

6.2 Conclusions

Since the invention of the pioneer DNA sequencer about half a century ago, the technology has progressed significantly. Unquestionably, the dideoxy method developed by Sanger and his colleagues is the gold standard in sequencing technology. The method revolutionized the field of genomics and other disciplines of biology that depend on DNA sequencing. In 2000 the first draft of the human genome was published using the Sanger method which took a lot of time and money. Later on, the field of sequencing witnessed an unprecedented change and revolution

marked by the development and commercialization of high-throughput and fast sequencing platforms known as next-generation sequencing technologies. The massively parallel sequencing characteristic of these platforms dramatically increased the size of output data concurrently decreasing the cost and time. Current NGS technologies offer dynamic applications in various fields of life through the analysis of biological sequences. In this book chapter, a brief outline of next-generation sequencing platforms, their chemistry, and vital insights gained through these technologies is discussed.

First NGS approach was based on the Roche's pyrosequencing which was followed by more advanced and robust platforms such as Ion Torrent/PGM, Illumina/Solexa, and ABI/SOLiD sequencing platforms. Each platform relies on unique sequencing chemistry and offers selective advantages (Table 6.2). The supremacy of high-throughput DNA sequencing technologies has been used by research community across the globe to solve and investigate diverse biological problems. The scale and efficacy of sequencing has redefined RNA and DNA sequencing fields and sheds light on how proteins interact with nucleic acids. NGS for whole genome, exome, transcriptome, methylome, metagenomics, ChIP, small RNA, *de novo*, and resequencing applications have limitless contributions in research.

Although the NGS approaches meet the requirement for DNA sequences sufficiently, they also have their disadvantages requiring improvements especially in data management and analysis. Sequence loading and accuracy of NGS need optimization and improvement. New sequencing platforms such as SMRT sequencing and nanopore sequencing are also emerging as new remarkable sequencing technologies with some obvious advantages and are often referred to as third-generation sequencing. These technologies mainly focus on approaches to overcome the flaws of the second-generation techniques. DNA sequencing has been evolving at a remarkable rate resulting in the development of better technologies. Despite the specific advantages and potential offered, all these platforms are challenged with technical flaws. But cutting-edge platforms, generations and resolutions, to address the existing difficulties will materialize in the near future.

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7 Annotation of Biological Network of Fungus *Saccharomyces cerevisiae* Using Cytoscape in Systems Biology

Prashant Ankur Jain, Ved Kumar Mishra, and Satyam Khanna

Abstract

Bioinformatics open software tool Cytoscape is worn for the visualizing and integrating gene expression of molecular interaction networks. Protein-protein interactions form the foundation for an enormous mainstream of cellular events, together with signal transduction and transcriptional regulation. It is implicit to swot analyze the interactions and communications among cellular macromolecules which are fundamental to the indulgent of biological systems. Interactions among proteins have been premeditated all the way during a number of elevated-throughput experiments. It has furthermore been predicted from side to side an assortment of computational process so as to leverage the immense quantity of sequence data which generate in the previous decade. We took into our approach an unfasten based software known as Cytoscape to integrate the biomolecular interaction networks among elevated throughput appearance data and shaped circular arrangement of a cell recitation over all genetic interactions. Circular arrangement of these biological complex pathways were grouped physically and were further categorized on the basis of starting point of their universal functions of indiscriminately selected IDs from SGD.

Keywords

Cytoscape · SGD · Biological network · BiNGO · jActive

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7.1 Introduction

Systems biology is endowed with an aim of junction of genomics, proteomics, metabolomics, and bio-computational modelling. Systems Biology is said to be the center of attention on experimental biological data (Sorger et al. 2005), computational biological techniques (Klauschen et al. 2007), and hypotheses testing (Kitano 2002). Systems biology is significant biochemical networks of biomolecules and molecular interactions (CajaMurcia 2006). Systems biology is hastily embryonic pitch of computer science, mathematics, physics, and biology. It is an accomplishment to study and identify composite biological systems besides captivating a synchronized included systems view with the approach of computational methodologies (Lodhi and Muggleton 2004). Systems biology is able to make available an explanation to the key problem such as unexplained inconvenience in cellular and molecular biology assortments, this science helps in curating sympathetic biological configuration as well as understanding biological classifications when viewed interms of calculation and implication in the multifaceted systems. Appearance profiling and significant proteomics have modernized biology by generating huge quantity of data about cell state. Genes by means of significant changes in appearance have instantaneous and extensive concentration as markers for diseases, stages of enlargement, and a variety of erstwhile cellular phenotypes. Genes among simultaneous appearance changes in excess of many circumstances are probable to be implicated in analogous functions or cellular processes; these genes often also share DNA sequence elements, providing evidence that they are regulated by common transcription factors. Analytical methods such as gene expression clustering (Eisen et al. 1998; Tamayo et al. 1999) and implication testing (Kerr and Churchill 2001; Rocke and Durbin 2001; Ideker et al. 2000), along with sequence ornamentation classification (Pilpel et al. 2001), contained requisite for enabling these researches and discoveries and briefing the data at each step.

For replica organisms, such as yeast, novel innovative technologies and data sets are easliy assembled and can be rearranged to answer simple questions (Altman and Raychaudhuri 2001). Orderly two amalgam screens and coimmune precipitation experiments are populating the unrestricted databases with thousands of protein-protein interactions and complexes (Uetz et al. 2000; Gavin et al. 2002). Estwhile uncompleted projects are essential huge numbers of protein→DNA interactions (Ren et al. 2000), along with protein microarrays are their assembly, it is probable now to map interactions among proteins and other tiny molecules (Zhu et al. 2001). These molecular interactions compose accessible and a well-positioned structure for understanding expression of genes and for integrating a spacious assortment of comprehensive state dimensions.

One origin of universal approaches to biological processes is the facts generated from genome sequencing and large-scale genetic analyses enlightening a previously vast range of interactions on the intensity of nucleic acids (Boone et al. 2007; Davierwala et al. 2005; Tong et al. 2004). Systems Biology research is somewhat addicted to genetic communications and networks and it further moves towards studying *Saccharomyces cerevisiae* (yeast), as a model organism which smoothly integrates practical genomics tools and consents well to systematic analyses overall. A universal perception of the topology of genetic communication networks in yeast has a broad significance, in view

of the fact that analogous networks are ordinary to be the cause of the association among genotype and phenotype in elevated eukaryotic species (Dolinski and Botstein 2005).

The emergence of systems biology at the same time as a novel regulation leaves several cell and molecular biologists incredulous (Kitano 2002). Molecular biology emphasizes reductionist approaches to suspiciously delineated inconvenience on the principle that significant insights originate from deep mechanistic understanding (Gibbs 2000).

7.2 Genetic Communication Bio-network Analysis

Genome sequencing and huge-range genetic analyses provides information on vast range of communications on the intensity of nucleic acids which is the key knoweledge to form the basis of understanding systemically the approaches to biological processes (Boone et al. 2007; Davierwala et al. 2005; Tong et al. 2004). On the whole of the insights into genetic communications and networks approach from studies, by means of the model organisim *Saccharomyces cerevisiae* (Yeast), wherein functional genomics tools consent to methodical analyses. A universal topology of genetic communication networks in yeast has an extensive significance, for the reason that analogous networks are anticipated to motivate the link between genotype and phenotype in higher eukaryotic species. Consequently, mapping genetic networks in representation organisms, for instance, yeast, provisions a significant framework for communications in supplementary complex systems (Dolinski and Botstein 2005).

Across-the-board analysis of genetic networks has presented a connection among the physical communication and the genetic communication networks. The physical-communication map (Gavin et al. 2002; Krogan et al. 2006; Ho et al. 2002), created by all-encompassing two-hybrid method or by affinity purification techique and subsequently using the mass spectrometry analyses, makes available a connection among genes and functional protein which are seen as multifaceted which in turn show their function as biochemical machines simultaneously. More willingly than physical information, the genetic communication map makes available functional information, largely identifying gene products that operate in functionally related pathways, in one such study (Parsons et al. 2004). In an additional illustration, synthetic-lethal genetic communications were encouraged for the identification of compounds having unambiguous pathways (Sharom et al. 2004). Ordinarily, genetic interaction understanding makes available an incredible quantity of principally expressive information and repeatedly sorting of data gives us a satisfactory view of related significant proteomic levels of each interactions (Schrattenhalz et al. 2004).

7.3 Materials and Method

The current project was carried out in Computational Biology, Dry lab at Rass Biosolution Private Limited. By means of observation and identifying interactions in model organism yeast, huge databases of protein-protein and protein-DNA interactions were cross referenced, which is important to study protein function,

evolution, and gene authoritarian dynamics. In the current research, we have integrated the interactions with mRNA expression data from the wet lab of Rass Biosolution Private Limited.

7.4 *Saccharomyces* Genome Database (SGD)

A scientific molecular biological and genetics database, *Saccharomyces* Genome Database (SGD) (www.yeastgenome.org), in recent times, created quite a few novel resources that consent to the assessment and amalgamation of information on a genome-wide range, enabling the user not only to find detailed information about individual genes but also to make connections across groups of genes with common features and across different species. The SGD was recognized to make available suitable resources for accessing the in haste expanding knowledge available for the growing *Saccharomyces cerevisiae* (yeast). SGD has as its most important objectives the prerequisite of information with reference to the DNA sequence and its entity components, RNAs, prearranged as well as encoded proteins along with the structures and biological functions of several identified gene products. An equally significant objective of SGD has been to develop online tools which have the same opinion to the user to effortlessly retrieve with display these types of information. This has outcome in graphical edges which are geared in the direction of biologists by means of the database, irrespective of their expertise by means of yeast. Beside expressive fragments of sequence, a gene name, a function and a role (e.g. enzymatic action), or a map point, one would be able to resourcefully doubt for information concerning a gene (Goffeau et al. 1996).

7.5 Software with Plug-ins

7.5.1 Cytoscape

Cytoscape is a bioinformatics software which is utilized for understanding molecular interacting networks which amalgamate through gene expression. Cytoscape was build up by the Institute of Systems Biology in Seattle in 2002. It was available online during July 2002 (V0.8). There are several supplementary plugins, accessible for use in network and molecular profiling examination by Cytoscape. This software can be downloaded via the link https://www.cytoscape.org, alongwith relevant to several classification of molecular sections along with interactions. Cytoscape allows the optical amalgamation of the network by means of profile expression, phenotypes, along with previous molecular state information, furthermore association of networks to online biological databases of functional annotations can be linked and parsed easily with it (Shannon et al. 2003; Bell and Lewitter 2006).

7.5.2 Network Nodes

The client decides on gene inventions commencing consumer-produced inventories, on the origin of GO (gene ontology) annotations, all genes consequent of a particular taxonomy ID, or else genes commencing a formerly saved Cytoscape network. While selecting genes during a user-defined catalogue, researchers are able to identify in their catalogues dissimilar identifiers commencing dissimilar databases by prepending their gene IDs through a prefix such as “RefSeq:” or “ORF:”

7.5.3 Edges/Interactions

Cytoscape sustains dissimilar kinds of communication databases to build biological networks: functional links to molecules contingent upon evolutionary methods (Prolinks (Bowers et al. 2004)); protein-protein, protein-DNA, and protein-RNA interactions (HPRD (authorization required) (Peri 2003), BIND (Gilbert 2005), BioGrid (Stark et al. 2006), and DIP (Xenarios et al. 2002); and metabolic pathway KEGG (Kanehisa 2002)). Consumers are able to choose databases and put database restrictions at this action of the network establishment wizard.

7.5.4 BioNetBuilder

This a plugin for Cytoscape for bio-network visualization. This plugin is vigorous to view scalable clarification for constructing and visualizing bio-networks for all types of genuses (Shannon et al. 2003).

7.5.5 BiNGO

BiNGO (Biological Networks Gene Ontology) plugin can be used both on a list of genes, posted as text, and interactively on subgraph of any selected biological networks to be visualized in Cytoscape.

7.5.6 jActiveModulus

jActiveModulus is a plug-in with the purpose of investigating a molecular interaction bio-network to discover expression-stimulated sub-network. This plugin is based on a technique, which is coined thorough statistical evaluation used for scoring sub-networks and it uses an investigative algorithm for finding sub-networks with high score (Ideker et al. 2002).

7.6 Results and Discussions: Landscape of Genetic Interactions

Multiple networks commencing SGD were loaded through software Cytoscape. A network accumulates every node along with edges that are encumbered. Cytoscape Simple Interaction File (.sif) format and Excel workbook (.xls) were reattributed as seen in Figs. 7.1 and 7.2.

The SIF format identifies nodes and interactions. These files are inured to import interactions when building a network for the unique IDs, in view of the fact that they are easy to generate in a text editor. Lines in the SIF files specify a source node, a relationship type, and one or more target nodes (Fig. 7.1). Cytoscape recognizes these extensions when browsing a directory for files of this type. Every node and edge in Cytoscape has an identifying name, most commonly used with the node and edge data attribute structures. The name of every node will be the name in this file by default.

Cytoscape has locally sustain for Microsoft Excel files (.xls). The table in these files contains network data and edge attributes (Fig. 7.2). The software reads these texts files and builds networks from them.

By using Cytoscape the files (Excel sheet) for all entries of yeast gene IDs, .sif extension file, .xls file, and gene ontology and genome annotation file were opened. Customization was done using VizMapper with the help of BioNetBuilder plug-in. BioNetBuilder plug-in draws a biological network pathway of the cell with their all genetic interactions (Fig. 7.3). Circular arrangement of these biological complex

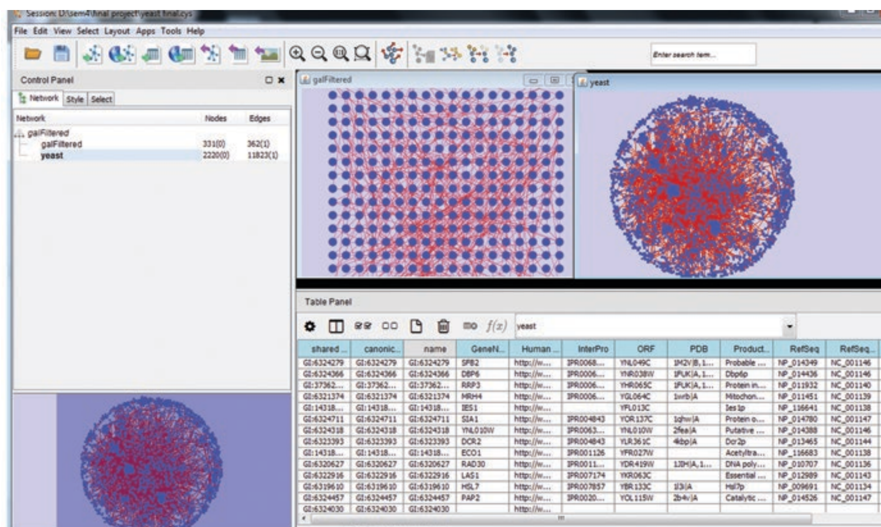

Fig. 7.1 Simple interaction file (.sif) downloaded for *Saccharomyces cerevisiae* (taxonomy ID: 4932)

	ID	Gene Nam	Inter Pro	ORF	PDB	Product N	Ref Seq	Ref SeqMI	UniProtKB	canonical Name
2	GI:632369	ERB1	IPR00168	YMR049C	1vyh|C	Erb1p	NP_01376	NC_00114	Q04660,S	GI:6323693
3	GI:632031	ALT2	IPR001176	YDR111C	1xi9|A	Alt2p	NP_01039	NC_00113	P52892,S5	GI:6320317
4	GI:632307	RPS0B	IPR001865	YLR048W	1K5X|B,1	Protein cc	NP_01314	NC_00114	P46654,S5	GI:6323077
5	GI:632337	RPP0	IPR001790	YLR340W	null	Conserve	NP_01344	NC_00114	P05317,R5	GI:6323371
6	GI:632301	PSR1	IPR004274	YLL010C	2hhl|A	Plasma m	NP_01309	NC_00114	Q07800,S	GI:6323019
7	GI:631947	RER2	IPR001441	YBR002C	1F75|A,1F	Cis-preny	NP_00955	NC_00113	P35196,S4	GI:6319474
8	GI:632518	YTA6	IPR003593	YPL074W	1E32|A,1x	Putative	NP_01525	NC_00114	P40324,P4	GI:6325183
9	GI:632102	DMC1	IPR001553	YER179W	1B22|A,1	Dmc1p	NP_01110	NC_00113	A38214,P	GI:6321027
10	GI:632007	CDC53	IPR001373	YDL132W	1LDJ|A,1L	Cdc53p	NP_01015	NC_00113	Q12018,S	GI:6320070
11	GI:632002	DLD1	IPR004113	YDL174C	1wvf|A	D-lactate	NP_01010	NC_00113	P32891,Q	GI:6320027
12	GI:632286	RSC4	IPR001487	YKR008W	1E6I|A,1J	Compone	NP_01293	NC_00114	Q02206,Q	GI:6322860
13	GI:632222	YIR035C	IPR002198	YIR035C	1AE1|A,1	Putative c	NP_01230	NC_00114	P40579,S4	GI:6322226
14	GI:632436	HUB1	IPR000626	YNR032C-	1M94|A	Hub1p	NP_01443	NC_00114	Q6Q546	GI:6324360
15	GI:632003	GLT1	IPR000583	YDL171C	1EA0|A,1	Glt1p	NP_01011	NC_00113	Q12290,Q	GI:6320030
16	GI:632309	XYL2	IPR002085	YLR070C	1DDA|A,1	Xyl2p	NP_01317	NC_00114	Q07993,S	GI:6323099
17	GI:632047	YDR266C	IPR001781	YDR266C	2gli|A	Protein of	NP_01055	NC_00113	Q05580,S	GI:6320472
18	GI:632159	RSR1	IPR001806	YGR152C	121P,1BK	GTP-bindi	NP_01166	NC_00113	A34511,P1	GI:6321591
19	GI:632222	LYS1	IPR007698	YIR034C	1pjc|A,2Q	Lys1p	NP_01230	NC_00114	P38998,S4	GI:6322225
20	GI:632205	FLX1	IPR001993	YIL134W	1okc|A	Flx1p	NP_01213	NC_00114	P40464,S4	GI:6322057
21	GI:632259	HOM6	IPR001342	YJR139C	1EBF|A,1	Hom6p	NP_01267	NC_00114	P31116,S3	GI:6322599
22	GI:631961	YBR139W	IPR001563	YBR139W	1AC5,1BC	Putative s	NP_00969	NC_00113	P38109,S4	GI:6319615
23	GI:632221	DAL4	IPR001248	YIR028W	1rzh|M	Allantoin	NP_01229	NC_00114	Q04895,S	GI:6322219
24	GI:632327	YEF3	IPR000357	YLR249W	2IW3|A,2	Translatio	NP_01335	NC_00114	DVBYE3,O	GI:6323278
25	GI:103837	NFS1	IPR000192	YCL017C	1ECX|A,1	Cysteine	NP_00991	NC_00113	P25374,S1	GI:10383773
26	GI:632291	PAM17	IPR013875	YKR065C	null	Pam17p	NP_01299	NC_00114	P36147,S3	GI:6322918
27	GI:632336	SGD1	IPR003890	YLR336C	null	Essential	NP_01344	NC_00114	Q06132,S	GI:6323368

Fig. 7.2 file in .xls format

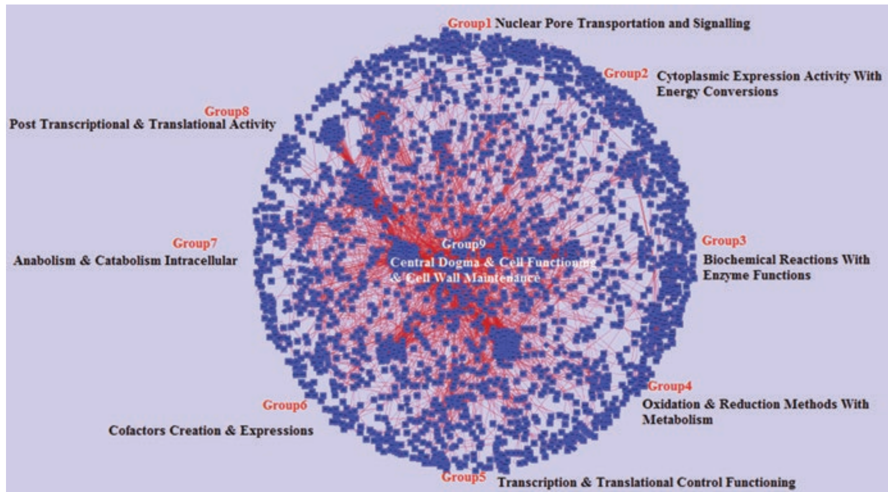

Fig. 7.3 Landscape of a cell describing all genetic interactions and process is annotated

pathways grouped physically and categorized on the starting point of their universal functions of indiscriminately selected IDs from SGD

The spherical arrangement intended for all genetic interactions in Figs. 7.4, 7.5, 6, 7.7, 7.8, 7.9, 7.10, 7.11, and 7.12 and Tables 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, and 7.9 shows the spaces of all the nodes in a rounded arrangement. It was expected that network surrounds 23,540 nodes along with 61,582 edges. This spherical

Fig. 7.4 **Group 1** Nuclear pore transportation and signaling

Fig. 7.5 **Group 2** cytoplasmic expression activity with energy conversions

arrangement separates the network into disconnected divisions autonomously. As a result the whole genetic interaction network was predicted to be divided into nine groups. Commencing the outcome we recognized translational genes which are dynamically contributing in one or further purpose later than the amalgamation of compound in the microbe.

Fig. 7.6 **Group 3** Biochemical reactions with enzyme functions

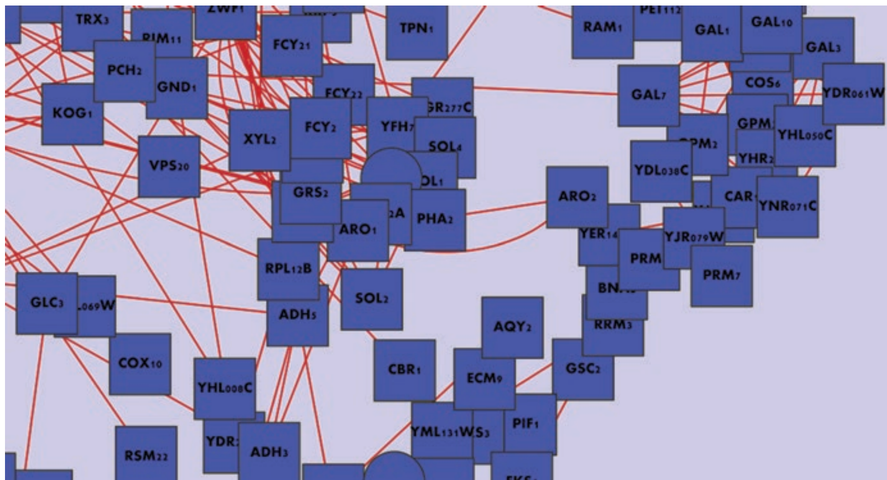

Fig. 7.7 **Group 4** Oxidation and reduction methods with metabolism

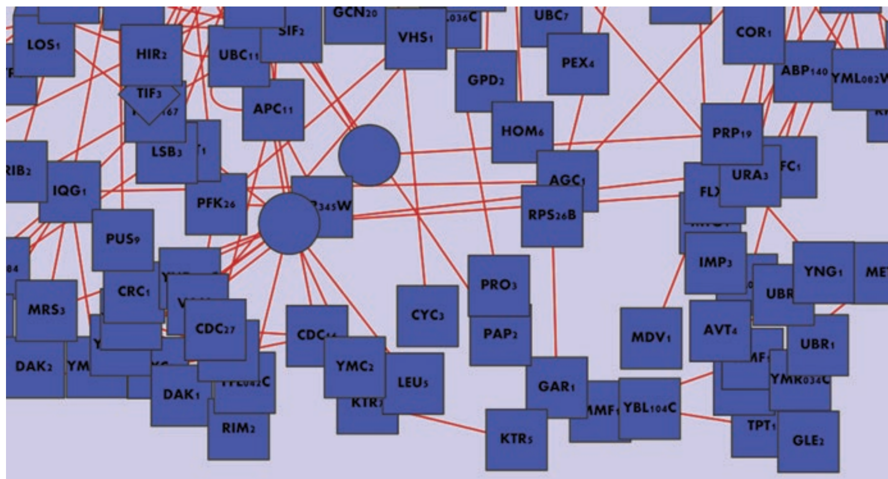

Fig. 7.8 **Group 5** Transcription and translational control functioning

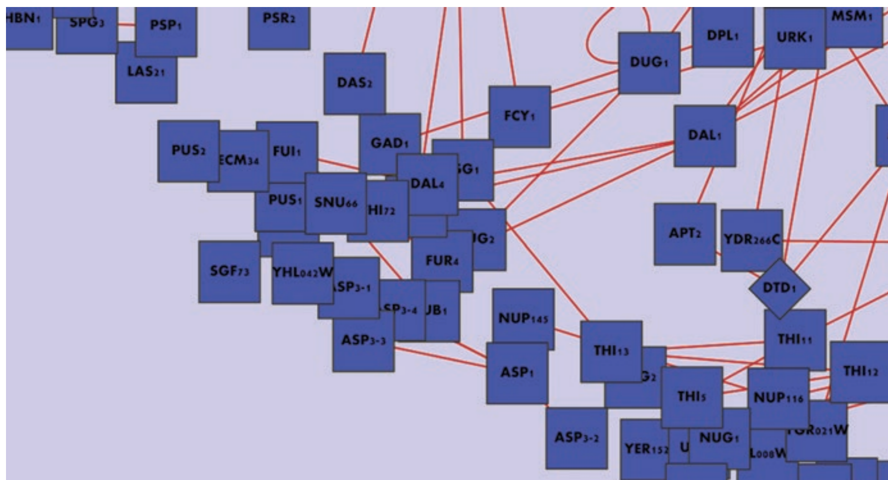

Fig. 7.9 **Group 6** Cofactors creation and expressions

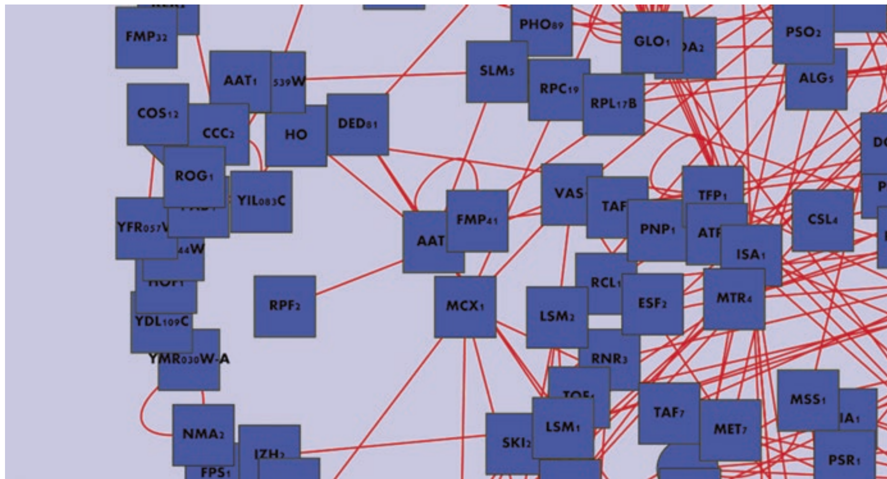

Fig. 7.10 **Group 7** Anabolism and catabolism intracellular

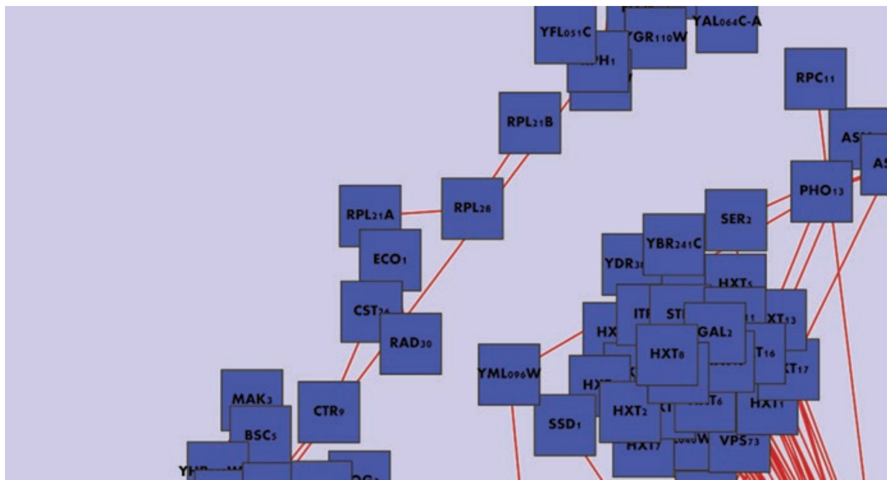

Fig. 7.11 **Group 8** Post transcriptional and translational activity

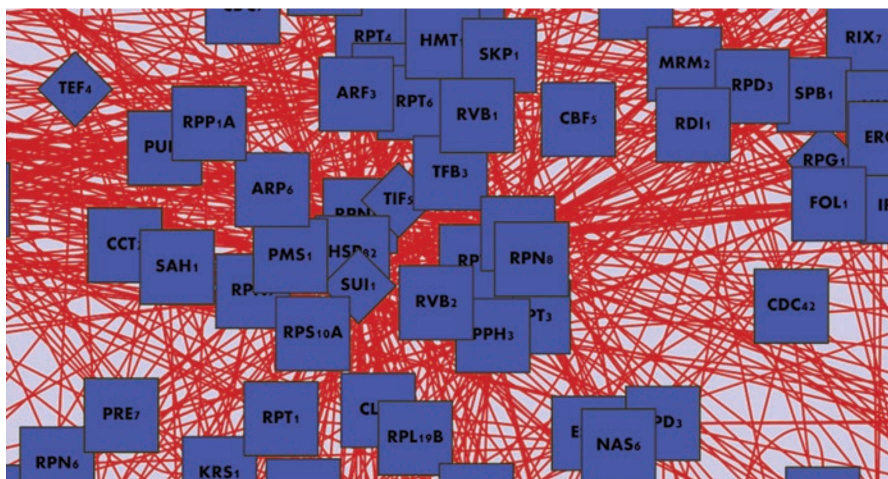

Fig. 7.12 **Group 9** Central dogma and cell functioning and cell wall maintenance

Table 7.1 Five different gene IDs and their functional expressions for group 1

Gene ID	Biological function	Molecular function	Cellular function
CDC21	Thymidylate synthase activity	dTMP biosynthetic process	dTMP biosynthetic process
YLR464W	Unknown	Unknown	Unknown
NPA3	ATPase activity GTPase activity	Mitotic sister chromatid cohesion, nucleocytoplasmic transport, RNA polymerase II complex localization to nucleus	Cytoplasm
AAD3	Aryl-alcohol dehydrogenase (NAD+) activity	Cellular aldehyde metabolic process	Unknown
AAD14	-SAME AS	SAME AS	Unknown

Table 7.2 Five different gene IDs and their functional expressions for group 2

Gene ID	Biological function	Molecular function	Cellular function
ERG7	Lanosterol synthase activity	Ergosterol biosynthetic process	Lipid particle
YKT6	Palmitoyltransferase activity, SNAP receptor activity	ER to Golgi vesicle-mediated transport, intra-Golgi vesicle-mediated transport, vacuole fusion, non-autophagic, vesicle fusion	Endosome, fungal-type vacuole, Golgi apparatus, membrane, SNARE complex
ROM1	Rho guanyl-nucleotide exchange factor activity	Activation of Rho GTPase activity, small GTPase-mediated signal transduction	Intracellular
FSP2	Oligo-1,6-glucosidase activity	Disaccharide catabolic process	Unknown
SPO75	Unknown	Ascospore formation, ascospore wall assembly	Integral component of membrane

Table 7.3 Five different gene IDs and their functional expressions for group 3

Gene ID	Biological function	Molecular function	Cellular function
ECM16	RNA helicase activity	Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA), ribosome biogenesis	Nucleolus, small-subunit processome
SAM50	Protein channel activity	Protein complex assembly, protein import into mitochondrial outer membrane	Integral component of mitochondrial outer membrane, mitochondrial sorting, and assembly machinery complex
YBT1	Bile acid-exporting ATPase activity	Bile acid and bile salt transport	Fungal-type vacuole
MIA40	2 iron, 2 sulfur cluster binding, oxidoreductase activity	Protein folding, protein import into mitochondrial intermembrane space	Integral component of mitochondrial inner membrane, mitochondrial intermembrane space
CDA2	Chitin deacetylase activity	Ascospore wall assembly	Chitosan layer of spore wall

Table 7.4 Five different gene IDs and their functional expressions for group 4

Gene ID	Biological function	Molecular function	Cellular function
FCY2	Cytidine transmembrane transporter activity, nucleobase transmembrane transporter activity	Cytidine transport, cytosine transport, purine-containing compound transmembrane transport	Plasma membrane
XYL2	D-xylulose reductase activity	Xylulose biosynthetic process	Unknown
GRS2	Glycine-tRNA ligase activity	Glycyl-tRNA aminoacylation	Cytoplasm
ARO2	Chorismate synthase activity, riboflavin reductase (NADPH) activity	Aromatic amino acid family biosynthetic process, chorismate biosynthetic process	Cytoplasm
PRM7	Unknown	Conjugation with cellular fusion	Integral component of membrane

Table 7.5 Five different gene IDs and their functional expressions for group 5

Gene ID	Biological function	Molecular function	Cellular function
YMC2	Organic acid transmembrane transporter activity	Mitochondrial transport, transmembrane transport	Mitochondrion
LEU5	Coenzyme A transmembrane transporter activity	Coenzyme A transport	Mitochondrial inner membrane
KTR5	Mannosyltransferase activity	Mannosylation	Golgi apparatus
GAR1	Box H/ACA snoRNA binding	rRNA processing, snRNA pseudouridine synthesis	Box H/ACA snoRNP complex, nucleolus, small nucleolar ribonucleoprotein complex
MDV1	Ubiquitin binding	Mitochondrial fission, mitochondrial genome maintenance, peroxisome fission	Mitochondrial outer membrane

Table 7.6 Five different gene IDs and their functional expressions for group 6

Gene ID	Biological function	Molecular function	Cellular function
APT2	Unknown	Unknown	Unknown
DTD1	D-leucyl-tRNA(Leu) deacylase activity, D-tyrosyl-tRNA(Tyr) deacylase activity	D-leucine catabolic process, D-tyrosine catabolic process, tRNA metabolic process	Cytoplasm
FUR4	Uracil/cation symporter activity	Transmembrane transport, uracil transport	Membrane raft, plasma membrane
SNU66	Unknown	Maturation of 5S rRNA, mRNA splicing, via spliceosome	U4/U6 x U5 tri-snRNP complex
PUS1	Pseudouridine synthase activity	snRNA pseudouridine synthesis, tRNA pseudouridine synthesis	Nucleus

Table 7.7 Five different gene IDs and their functional expressions for group 7

Gene ID	Biological function	Molecular function	Cellular function
FMP32	Unknown	Unknown	Mitochondrian
AAT1	L-aspartate:2-oxoglutarate aminotransferase activity	Asparagine biosynthetic process from oxaloacetate, aspartate biosynthetic process, chronological cell aging, replicative cell aging	Mitochondrion
COS12	Unknown	Unknown	Unknown
ROG1	Lipase activity	Cellular lipid metabolic process	Unknown
RPF2	5S rRNA binding, 7S RNA binding, rRNA binding	Assembly of large subunit precursor of preribosome, maturation of 5.8S rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA), maturation of LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA),	
Ribosomal large subunit assembly	Nucleolus		

Table 7.8 Five different gene IDs and their functional expressions for group 8

Gene ID	Biological function	Molecular function	Cellular function
RPL28	RNA binding, structural constituent of ribosome	Cytoplasmic translation	Cytosolic large ribosomal subunit, nucleus
ECO1	Acetyltransferase activity, chromatin binding	Chromosome organization, DNA repair, DNA replication, double-strand break repair, establishment of mitotic sister chromatid cohesion, internal peptidyl-lysine acetylation, mitotic chromosome condensation, regulation of DNA replication, regulation of mitosis, telomere organization, tRNA gene clustering	Nuclear chromatin, nuclear replication fork
RAD30	DNA-directed DNA polymerase activity	Error-free translesion synthesis, error-prone translesion synthesis	Mitochondrion, nucleus, replication fork
CTR9	RNA polymerase II C-terminal domain phosphoserine binding, RNA polymerase II core binding, RNA polymerase II transcription factor binding transcription factor activity, triplex DNA binding	DNA-templated transcription, termination, mRNA 3′-end processing, positive regulation of histone H3-K36 trimethylation, positive regulation of phosphorylation of RNA polymerase II C-terminal domain serine 2 residues, positive regulation of transcription elongation commencing RNA polymerase I promoter, regulation of chromatin silencing at telomere, regulation of histone H2B conserved C-terminal lysine ubiquitination, regulation of histone H3-K4 methylation, regulation of transcription by chromatin organization, regulation of transcription initiation commencing RNA polymerase II promoter, regulation of transcription involved in G1/S transition of mitotic cell cycle, regulation of transcription-coupled nucleotide-excision repair, snoRNA 3′-end processing, snoRNA transcription commencing an RNA polymerase II promoter, transcription elongation commencing RNA polymerase I promoter, transcription elongation commencing RNA polymerase II promoter, transcription commencing RNA polymerase I promoter	Cdc73/Paf1 complex, nucleus, transcriptionally active chromatin
RPL21B	Structural constituent of ribosome	Cytoplasmic translation	Cytosolic large ribosomal subunit

Table 7.9 Five different gene IDs and their functional expressions for group 9

Gene ID	Biological function	Molecular function	Cellular function
RPP1A	Protein kinase activator activity, structural ingredient of ribosome	Cytoplasmic translation, positive parameter of protein kinase movement	Cytosolic large ribosomal subunit
PMS1	ATP binding, ATPase activity, *contributes to* dinucleotide insertion or deletion binding, *contributes to* DNA insertion or deletion binding, *contributes to* double-stranded DNA binding, *contributes to* loop DNA binding, *contributes to* single-stranded DNA binding	Meiotic mismatch repair, mismatch repair	MutLalpha complex
SUI1	Ribosomal small subunit binding, translation initiation factor activity, translation initiation factor binding	Formation of translation preinitiation complex, maintenance of translational fidelity	Eukaryotic 43S preinitiation complex, multi-eIF complex
NAS6	Unknown	Proteasome regulatory particle assembly, proteolysis	Cytosol, nucleus, *colocalizes with* proteasome regulatory particle
MRM2	rRNA (uridine-2′-O-)-methyltransferase activity	rRNA methylation	Mitochondrion

7.7 Conclusion

Computational processes approximating categorization and network-based algorithms are up to be used to be aware of the manner of accomplishment and the usefulness of a given compound. This will have need constructing an essential perceptive of systems biology that underlies ordinary biological pathological and processes, and the enlargement of novel technologies that will be vital to accomplish this objective. Cytoscape is an open-source software for viewing and alalyzing huge range amalgamated molecular interaction network data. The Cytoscape interior explanation of essential description, for instance, network arrangement and mapping of data, attributes to visual demonstrate properties. The multifaceted genetic background and interaction networks were analyzed to considerably recognize the response of the compound. In the vicinity of prospect, the generally grave assignment is to investigate our identified sub-networks in the laboratory. Because large interaction networks are suspected to contain many false positives, an initial experiment would be to verify that the interactions in each sub-network are reproducible and present under the subnet's particular set of conditions.

Protein-protein interaction networks make available an easy general idea of the network of communications consequently at the same time as to obtain consign inside a cell. The immense amounts of sequence data quantities consequently have been generated. It also has been leveraged to construct enhanced predictions of communications and functional links between proteins, over and above individual protein functions. By integrating investigational process intended for influential protein-protein interactions and computational methods for prophecy, many constructive and functional data on protein-protein interactions are generated, together with a number of far above the ground eminence databases.

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Recent Advances in Microbial Genome Sequencing

8

Rajpal Srivastav and Garima Suneja

Abstract

Microbes play significant roles in environmental ecological balance and human health. Microbes are associated with infection and immunity in humans. The microbial culturing and analysis has improved over the course of period with the advent of DNA sequencing technology. Since first bacterial genome of bacterium *Haemophilus* was sequenced by using Sanger method almost 20 years back, DNA sequencing technology has advanced drastically in capability and applications. With advances in next-generation sequencing technology involving improvement in the chemistry, increase in the output and quality of data, microbial genome sequencing has become an affordable approach to do comprehensive microbial analysis. The next-generation sequencing methods are more advanced and data throughput in a typical NGS method is about 100-fold higher compared to Sanger sequencing. The next-generation sequencing technology has revolutionized fields of genetics, genomics, microbiology, and clinical microbiology. Now whole genome microbial sequencing can be performed within few hours. With the third-generation sequencing like single-molecule sequencing, the possibility of having more of complete microbial genomes has increased. Analysis of complete genome sequences would provide a great insight about microbial diversity, virulence, evolution of microbes, and host-pathogen interactions. In this chapter, the recent advancements in the field of microbial genome sequencing have been discussed.

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Microbial genome · Metagenomics · Whole genome sequencing · Next-generation sequencing

8.1 Introduction

Microbes form a significant portion of biomass on the planet. These microbes are also an indistinguishable part of the human body and have roles in infection and immunity. Despite technological advances in recombinant DNA technology and microbial culturing, we are still unable to culture all existing microorganisms in laboratory settings. A possible reason for this is unique cultivation conditions like temperature, nutrition levels, microbial interactions, and fastidious growth conditions, which are difficult to maintain in laboratories. Moreover, the conventional techniques of microbial culturing and analysis are laborious, time-consuming, and sometimes biased because more than 90% of microbes are unculturable. Metagenomics has resolved this problem as the community DNA can be analyzed without the requirement of culturing microorganisms. This technique also provides a complete profile of the microbial communities in any environment. The advent of DNA sequencing methods with their improved and cost-efficient technologies has further led to the advanced analysis within short frame of time.

The first bacterial genome of *Haemophilus influenzae* was sequenced in 1995. Since then, DNA sequencing technologies have evolved rapidly in capability and applications. These advancements led Human Genome Project to deliver the first draft of the human genome sequence in (2001) (Craig Venter et al. 2001). In 2006, next-generation sequencing (NGS) techniques emerged to provide an unbiased means to examine billions of templates of nucleic acids at a rapid pace. Currently, microbial genome sequences derived from isolates as well as metagenomes hold importance in characterizing microbes for generic and specific functions, niche adaptations, evolution, and strain-level differentiations. In this chapter, the recent advancements in the field of microbial genome sequencing have been discussed.

8.2 Conventional Microbial Genome Sequencing

The conventional methods to identify microbes by culturing and phenotypic approaches are inexpensive. These can be used in normal lab conditions to easily characterize the microbes and microbial pathogens. However, such methods are laborious and time consuming. Further, molecular characterization is being used for identification purposes. Later, the sequencing of sub-cloned 16S amplicons and genomic fragments complemented the culturing techniques. Before 1995, the bacteriophage lambda genome of size of 48,502 base pairs was sequenced with a random strategy. However, this conventional microbial sequencing and its analysis was

a cumbersome and costly affair. Now, advanced methodologies are followed to overcome these drawbacks.

Advancements in DNA sequencing technologies with next-generation sequencing (NGS) have now revolutionized every aspect of microbial genome sequencing. The advent of multiple displacement amplification reaction (MDA), which amplifies a single microbial genome by a billion-fold, makes next-generation sequencing a powerful tool in microbial genomics (Lasken 2007). The first bacterial genome sequenced almost two decades back using Sanger sequencing was *Haemophilus influenzae* (Fleischmann et al. 1995). The Sanger sequencing method was the simplest and widely used sequencing method. These techniques are also referred as *first-generation sequencing technology*. The systematic procedure of Sanger sequencing includes preparation of shotgun library, isolating sequencing templates, performing sequencing reactions, and finally performing capillary electrophoresis. This technology can sequence fragments up to 1 Kilo-base pairs (Kb) with 99.99% accuracy. The output of the method is assembled to generate draft genomes. Since the first draft of a microbial genome, many organisms have been sequenced at a very rapid pace. There is a steep increase in the numbers of the draft bacterial genomes and it is continously increasing.

Sanger sequencing was advantageous and used to provide useful information in comparison to traditional microbial culturing analysis. Earlier, the first bacterial genome of *Haemophilus influenzae* by Sanger sequencing took more than 1 year (Fleischmann et al. 1995). Sanger sequencing is a technically laborious and time-taking procedure. Further, there were specific technical requirements for Sanger sequencing methods. Therefore, it is not possible for general laboratories to perform these sequencing reactions. Thus, majority of bacterial genome sequencing projects were restricted to a few large sequencing laboratories. Another limitation is that *de novo* sequencing is not possible using Sanger sequencing methods. The limitations of conventional Sanger sequencing were overcome by more advanced sequencing technologies, which have been discussed in the following section.

8.3 Advances in Microbial Genome Sequencing

8.3.1 Next-Generation (Second-Generation) Sequencing

The *next-generation sequencing* (NGS) is a more advanced sequencing technology compared to conventional Sanger sequencing. The data throughput is 100-fold higher compared to Sanger sequencing (Pareek et al. 2011; Grada and Weinbrecht 2013). It is also referred as *high-throughput genome sequencing* (HTGS) because of such huge amount of sequencing data generated (Liu et al. 2012). Millions of DNA molecules are sequenced together in parallel in a typical NGS reaction. NGS was introduced in year 2000 using 454 (Roche) with pyrosequencing approach. A typical workflow for next-generation genome sequencing includes steps like microbial sample collection, nucleic acid extraction, genomic DNA fragmentation, adapter addition, DNA Library preparation, and sequencing followed by data analysis

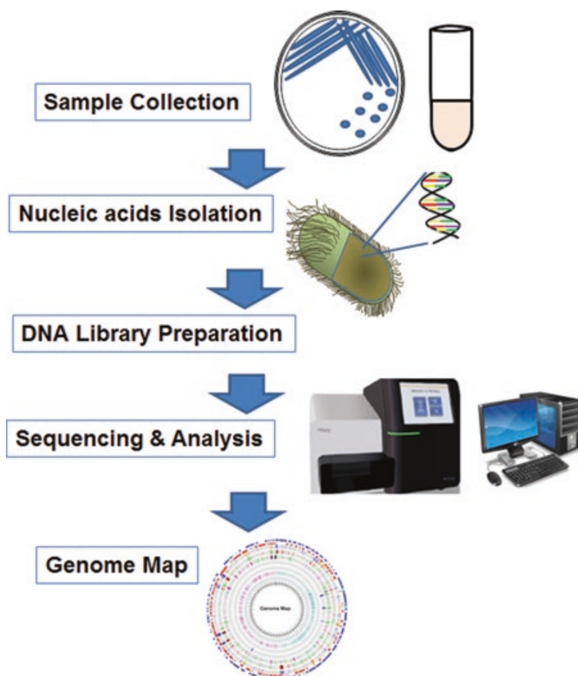

Fig. 8.1 The simplest schematic representation of workflow of microbial genome sequencing depicting the major steps during the procedure

(Fig. 8.1). The entire process of a typical NGS experiment from the microbial colony harvest to acquisition of analyzable data takes less than 60 hours depending on the sequencing platform.

There has been tremendous increase in the pace of microbial research with new advanced NGS technologies. Further, the decreasing cost of the technology has propagated microbial genome sequencing tremendously. There are various commercial platforms available to perform high-throughput next-generation sequencing like 454, Illumina, Ion Torrent, ABI SOLiD, etc (Pareek et al. 2011; Liu et al. 2012; Grada and Weinbrecht 2013). Two main NGS platform methods are currently used, namely, short read platforms (including Illumina and Ion Torrent). Illumina platforms have HiSeq2000 and MiSeq that perform an ultra-high-throughput analysis. These commercial platforms have difference in their output, read length, and coverage (Buermans and Dunnen, 2014). The limitation of these sequencing methods is its reduced sequence length and quality, though high throughputs balance for its reduced lengths. Each platform has its own advantages and limitations. The choice of use of these platforms for microbial genome sequencing depends on the requirements of the analysis, throughput, and desired applications.

8.3.2 Third-Generation Sequencing

There were some technical issues with second generation of sequencing like short read length (30–450 bases), errors due to short read lengths, and laborious sample preparation methods for some platforms. To overcome these drawbacks, more

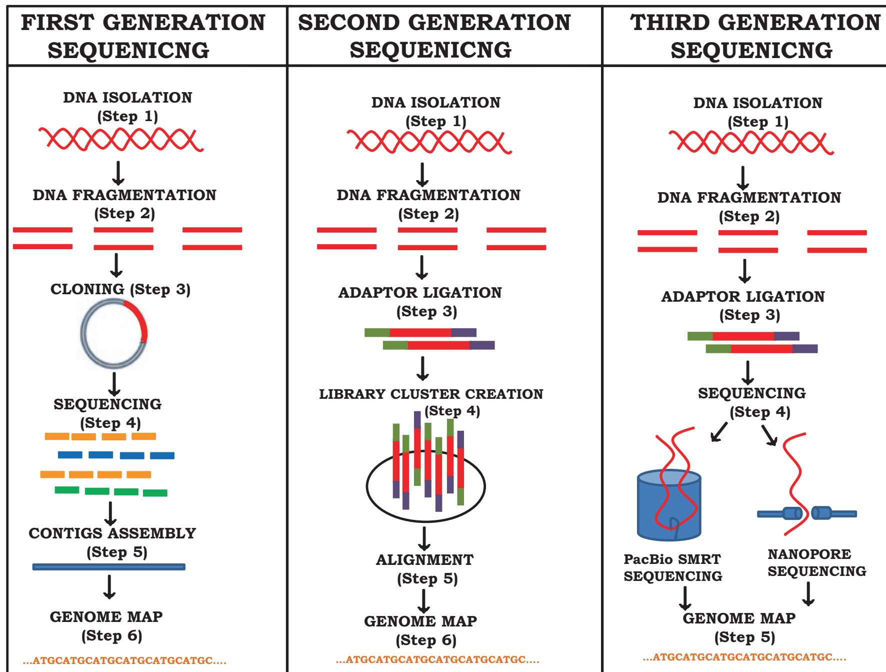

Fig. 8.2 The schematic representation and comparison of workflow of the first-, second-, and third-generation sequencing technologies

advanced sequencing platforms have been developed. These are rapid and yield reads up to 20 Kb for each DNA molecule, called as *third-generation sequencing* technology (Coupland et al. 2012; Buermans and Dunnen 2014). There are broadly two categories; first is single-molecule real-time sequencing (Pacific Biosciences), and second type is nanopore sequencing (Oxford Nanopore) under third-generation sequencing technology. The methodology and working principle of these third-generation sequencings is different from earlier sequencing methods (Fig. 8.2).

During *single-molecule real-time (SMRT) sequencing,* single-molecule of DNA is detected per reaction during real time, whereas the basic principle of *nanopore sequencing* is to measure changes in electrical properties as biomolecules such as DNA translocate through the pore and then to use electrical changes to identify the exact DNA base. These advanced third-generation sequencing technologies such as PacBio and MinION can produce much longer reads of several thousand base pairs compared with the first- and second-generation sequencing technologies. These third-generation sequencing methods can be used for direct DNA and RNA sequencing, real-time data acquisition and analysis, long reads, and *de novo* assemblies of repeated sequences and complex regions but at the cost of read quality (Coupland et al. 2012; Buermans and Dunnen 2014).

8.4 Classification of Advanced Commercial Sequencing Platforms

With the advancement in NGS technology, there are many commercial platforms available for microbial genome sequencing like Illumina, Ion Torrent, Applied Biosystems SoLiD and polonator platforms, Roche 454 platforms, Pacific Biosciences platforms, Oxford Nanopore platform, etc. These platforms have differences in their sequencing chemistry, detection method, and molecular numbers during reaction. These available sequencing platforms can be generally classified on the following basis.

8.4.1 Based on Sequencing Chemistry

- *Sequencing by synthesis* – This includes use of polymerase to drive synthesis reaction, where products of the reaction are measured to yield sequencing data, e.g., Illumina, Ion Torrent, Roche 454 platforms, and Pacific Biosciences platforms.
- *Ligation-mediated synthesis* – This includes ligation-mediated synthesis and products of the reactions are measured, e.g., Applied Biosystems SoLiD and polonator platforms.
- *Direct measurement of DNA molecule* – This includes directly measuring the DNA molecules property to yield sequencing data, e.g., Oxford Nanopore platform.

8.4.2 Based on Detection Method

- *Optical detection* – This method detects optical property to make sequencing base calls like detection of fluorescently modified nucleotides, e.g., Illumina and Pacific Biosciences platforms. Roche 454 platform detects light via pyrosequencing.
- *Non-optical detection* – This method includes detection using non-optical properties like detection of H^+ release during a polymerization reaction via solid-state sensor, e.g., Ion Torrent. Oxford Nanopore platform also uses non-optical detection method. It detects translocation of DNA through a nanopore sensor.

8.4.3 Based on Molecule Number

- *Detection of amplified DNA* – This includes detection of the clonally amplified DNA molecules Illumina, Ion Torrent, and Roche 454 platforms.
- *Single-molecule detection* – In this method, the detection of single DNA molecule per reaction, well, or sensor is performed, e.g., Pacific Biosciences and Oxford Nanopore platforms.

Table 8.1 The characteristics of different sequencing platforms (data obtained from manufacturers' websites)

Platforms	Manufacturer	Sequencing chemistry	Read length (bp)	Throughput (Gb)
NextSeq	Illumina	Sequencing by synthesis-reversible terminator	2×250	10–120 Gb
MiSeq	Illumina	Sequencing by synthesis-reversible terminator	2×300	0.3–15 Gb
Hiseq 3000	Illumina	Sequencing by synthesis-reversible terminator	2×150	660–750 Gb
Hiseq 4000	Illumina	Sequencing by synthesis-reversible terminator	2×150	1300–1500 Gb
HiSeq X	Illumina	Sequencing by synthesis-reversible terminator	2×150	1.6–1.8 Tb
ion Torrent	Life Technologies	Sequencing by synthesis-H^+ ions detection	400	2 Gb
Ion Proton	Life Technologies	Sequencing by synthesis-H^+ ions detection	200	10 Gb
SOLID 5500xl	Life Technologies	Fluorescent probe ligation	50×50 paired end	320 Gb
Sequel	Pac Biosciences	Single molecule real time	up to 30 kb	20 Gb
PacBio RSII	Pac Biosciences	Single molecule real time	4200–8500	275–375 Mb
454 GS Junior	Roche	Pyrosequencing	600–700	35–53 Mb
454 FLX +	Roche	Pyrosequencing	1000	700 Mb
PromethION	Oxford Nanopore	Ion current sensing	up to 100 kb	4000–7000 Gb
MinION	Oxford Nanopore	Ion current sensing	up to 100 kb	0.1–1 Gb
Heliscope	Helicos	Single molecule real time	30 bp	15 Gb

These sequencing platforms have similarities as well as differences depending on the chemistries and detection methods (Ashkenasy et al. 2005; Lundquist et al. 2008; Buermans and Dunnen 2014) (Table 8.1). These differences lead to different strengths and weaknesses among the platforms. Therefore, it is advisable to use multiple platforms in a single experiment, with the goal of capitalizing on the strengths of each platform. The common parameters used to compare platforms are sample preparation time, number of reads produced, the length of reads, cost per run, run time, and finally total cost.

8.5 Advancement in Read Length and Data

Since the first sequencing event, the revisions to read lengths are occurring rapidly as chemistries of sequencing reactions are being improved. Despite increase in read length, the highest-output platforms of the second-generation continue to have relatively short read lengths (35–300 bases per read), e.g., Illumina HiSeq, MiSeq, or

NextSeq series for genome sequencing. Paired-end sequencing (up to 2 × 300 bp for the MiSeq platform) and the depth compensates for short read lengths in these sequencing methods.

Third-generation technologies like Pacific Biosciences platform has a long-read technology, and it produces about 880,000 reads per 16 single-molecule real-time cell instrument run with read length averages about 10,000 bases. The sequencing reaction occurs on the SMRT bell template, detected with a zero-mode waveguide optical system and in SMRT bell, a strand-displacing polymerase can sequence the template several times providing multiple reads of each base and thus increases the accuracy of reads. In 2015, Pacific Biosciences' new platform named Sequel has a significant change from the original instrument, PacBio RSII, in both form and capabilities, with a substantial increase in read density compared with the available RSII, with each SMRT cell having 1 million zero-mode waveguides (compared with 150,000 on the RS II), increasing the read output by approximately seven times. This approach provides application in microbial genome sequencing and molecular analysis (Ashkenasy et al. 2005; Lundquist et al. 2008; Buermans and Dunnen 2014).

With the advancement in technology, various sequencing platforms have been improved for high-throughput data and speed. Pacific Biosciences platform, the Sequel, provides about 6X data output, while Oxford Nanopore has PromethION. Ion Torrent's third iteration sequencer (Ion S5 and Ion S5 XL) has local computing with improved analysis speed. The Illumina platforms have HiSeq X and NovaSeq platforms, which provide ultra-high output. The Illumina platforms can generate highly accurate data and be used with any species with minimum 30X targeted coverage.

8.6 Applications and Scope of Microbial Genome Sequencing

8.6.1 Rapid Microbial Characterization

The microbial genome can be used for sequencing or *de novo* sequencing based on its further application and analysis. The variant sequencing called *amplicon sequencing* helps in rapid microbial identification, taxonomy, and diversity analysis. Amplicon sequencing is a highly targeted approach that enables analysis of variations in specific 16S rRNA gene regions. The unique features of amplicon sequencing include efficient analysis and high coverage by multiplexing of thousands of amplicons in a reaction. The analysis (certain variable regions of 16S rRNA) may favor amplification of a broader spectrum of bacteria than others may, and its resolution is limited to genus or higher taxonomic levels.

In order to compare and differentiate among different strains of a species, *whole genome sequencing* (WGS) is a more comprehensive approach. It can analyze and classify numerous genomes rapidly and is a better alternative to amplicon sequencing. Whole genome sequencing approaches allow detailed functional analysis and characterization of microbial communities (Oniciuc et al. 2018). The bacterial genome reads can be aligned to reference genomes for assembly or *de novo* assembly of contiguous sequences is done for gene predictions. For microbial identification

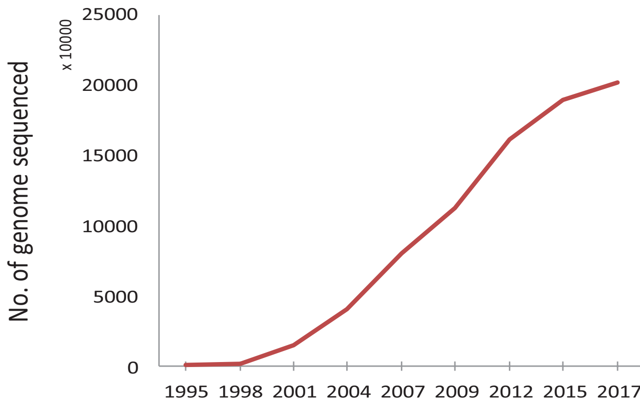

Fig. 8.3 The number of genomes sequenced year-wise and submitted to NCBI till year 2017

purpose, an assembled bacterial genome is aligned to a closely related reference genome (determined by 16S identity) followed by *in silico* genome–genome comparison. WGS sequencing has become an efficient tool to examine the protein-coding regions of microbial genomes and hence their functions (Fraser et al. 2000). In the last decade, the number of draft microbial genomes has increased many folds with improvements in NGS technologies which provide high sequencing depth (Fig. 8.3). However, the complete genome sequence numbers are not increasing with similar pace. In such situations, WGS with third-generation sequencing technology would provide more comprehensive information about microorganisms. It is expected that third-generation sequencing would change the concept of draft microbial genomes all together as more accurate and complete genome data would be obtained.

8.6.2 Advances in Pathogen Detection

The availability of low-cost genome sequencing has resulted in a broader use of WGS, including metagenomic studies and pathogen detection in clinical microbiology. Foodborne pathogens like bacteria and viruses cause diseases either directly (by infectious agents) or indirectly (by toxic metabolites) and can have drastic health and economic problems in developing as well as in developed countries. Loman NJ and his team reported that MiSeq had the highest throughput run as compared to Ion Torrent PGM and 454 GS in sequencing of *Escherichia coli* O104:H4 (Loman et al, 2012). Also, it has been reported that sequencing libraries made using Illumina Nextera XT produced more accurate multi-locus sequence type in less time and cost as compared to HiSeq and Ion Torrent (Perkins et al, 2013). HiSeq series allow large DNA parallel sequencing at reduced cost, while MiSeq is useful for smaller projects.

These high-throughput sequencing technologies are widely used for detection of pathogens in clinical microbiology (Balloux et al 2018). The WGS approaches have improved the diagnosis of bacterial, fungal, and viral pathogens rapidly and

comprehensively. WGS sequencing studies are efficient for slow-growing pathogens like *Mycobacterium* spp. It is useful for characterization of mixed infections containing samples from brain abscess, lymph node biopsy, mastoid abscess material, etc. The pathogenicity of microbes can be analyzed using genome comparison. The comparative genomic analysis performed among 30 *Staphylococcus aureus* genomes, 15 *Staphylococcus argenteus*, and 6 *Staphylococcus schweitzeri* genomes revealed that all three species had rare core genome with interspecific recombination (Zhang et al. 2017). There is no doubt that next-generation sequencing would play a significant role in research, health care, and industrial experiments and that the number of available applications will continue to grow with the innovation and creativity of the scientific community.

8.6.3 Understanding Microbial Evolution

The understanding of microbial evolution is important as microbes are important component of human health, and there are about 1.3 times more microbes in human body than number of human cells. The microbes evolve along with its host and adapt to changes in its microenvironment (Stappenbeck et al. 2002; Ley et al. 2006). Various microbes are diverse at the strain level depending upon its habitat. The gut bacteria produce various antimicrobial molecules like cathelicidins and defensins, which maintains the homeostasis in gut microbial populations (Hooper and Gordon 2001). The faster and cheaper NGS technologies especially whole genome sequencing made microbial genome analysis easy. Whole genome sequencing provides information about microorganisms' adaptability and evolution. Whole genome sequence analysis of *Staphylococcus aureus* revealed mobile genetic elements that are responsible for evolution of methicillin resistance (Ali et al. 2019).

Single-genome sequencing methods provide information about virulence and host-microbial interaction factors. Further, the high-throughput sequencing technologies can provide information about secondary metabolites released by microbes. The detailed phylogenetic analysis of microbes can be performed by HTP-NGS. Now, microbiome analysis has become easier, and the understanding about microbes and their evolution is increasing rapidly with use of next-generation sequencings.

8.6.4 Metagenomics Advances

There have been various improvements in the genomics analysis with the involvement of next-generation sequencing. This complete profile of the sample's entire DNA can be explored using *de novo* sequencing and metagenomic analysis. *Metagenomics* is a branch of genomics where microbial community DNA is directly analyzed without culturing the microorganisms (Handelsman 2004; Schloss and Handelsman 2005; Wooley and Ye 2009; Wang et al. 2015). The advantage of this analysis is that it avoids the need of culturing the microbes. There are microorganisms in some ecological niches, which are difficult to culture because of specific

growth conditions. The general scheme of a typical metagenomics analysis includes isolation of DNA from an environmental sample, fragmentation of DNA, and using it for sequence or function-based analysis (Handelsman 2004; Ghai et al. 2012; Mizuno et al. 2015; Wang et al. 2015).

The functional analysis requires cloning DNA into a suitable vector to create a metagenomic library, sequencing the clonal fragments followed by NGS analysis. The library can be screened and analyzed for particular properties or novel characteristics like salt tolerance, pH resistance, antibiotics' resistance, or specific enzymes (Handelsman 2004; Wang et al. 2015; Ranjan et al. 2018). Alternately, direct DNA sequencing of the DNA fragments exclusive of the cloning step can be used for functional analysis or for recovery of genomes from uncultured microbes of the sample.

Another usefulness of metagenomics is in clinical microbiology to detect the cause of the infection. The analysis of all the DNA of infected sample in comparison to control sample would provide the information about the infectious agent. The integration of NGS into metagenomics provides a rapid analysis of complex ecological niches, which is otherwise a time-consuming and laborious task. Using DNA-based sequencing, we can analyze the complexity and constitution of an entire ecosystem (Buermans and Dunnen 2014). Metagenomics now can provide answers to many questions like consequences of environmental changes and cause of the changes. The progress in the human gut microbiome analysis has been improved by the use of next-generation sequencing with metagenomics approach. It has provided very interesting insights about the relationship between human health and gut microbes. The health hazards like obesity and other diseases are associated with the human gut microbiome (Ley et al. 2006; Salzman et al. 2007; Wang et al. 2015).

8.6.5 Advances in Computational Approaches

The generation of huge amount of data in these high-throughput sequencing technologies necessitates parallel advances in computation analysis. There are various online softwares as well as offline tools to support post-process analysis. During a NGS reaction, millions of short reads are generated, which need to be assembled into manageable data. There are various assemblers like Velvet, Ray, and ABySS, which can assemble gigabytes data into 10s and 1000s of contigs of genome and metagenome, respectively (Zerbino and Birney 2008; Paszkiewicz and Studholme 2010). These assemblers can be used for reference-based and *de novo* assembly of the genomes. Reference-based assembly is performed when there is availability of reference genomes in databases, to be used to order the contigs. This set of assemblies may have some degree of biasness due to limitation of existing databases. Therefore, for unique environment analysis, the raw reads can be assembled into contigs *de novo*. Assemblers use greedy-graph algorithm, overlap-layout-consensus algorithm, or de Bruijn graphs to generate contigs. Some of the softwares commonly used for genomic and metagenomic assembly include Velvet, MetaVelvet, ABySS, SOAP, SPAdes, Ray Meta, Meta-IDBA, MIRA4, MetaAMOS, and Newbler (Zerbino and Birney 2008; Paszkiewicz and Studholme 2010; Namiki et al. 2012; Treangen et al. 2013). *De novo* assembly is best suited while exploring unique

microbial populations or unique environments, whereas reference mapping is best suited when resequencing to complete draft genomes or for pathogen identification and pathovar differentiation. The use of assembly depends on computational memory constraints, biological complexity of the data, availability of reference genomes, and application.

There may be some other types of biasness during assembly of contigs owing to low coverage or presence of repetitive sequences. Hence, longer contigs and higher read coverage should be considered. Consequently, precise analyses of assembly metrics such as N50, average coverage, and total assembly size are used to measure the efficiency of a good assembly. Multiple algorithms are being scripted every day to meet the need for NGS applications. There is need for a large memory and specific hardware for computation and storage of NGS data. The algorithms need to be designed with respect to a specific objective. There are improvements of data visualization tools in complete data analysis. However, further improvements are a prerequisite with constantly increasing high-throughput sequencing.

8.7 Conclusion and Perspective

There have been several advances in next generation sequencing technology like improvement in the chemistry, flow cells on the platform, and increase in the output and quality of data. These advancements have paved way for an increased understanding of genomics, epigenomes, epi-transcriptomes, metagenomes, etc. The cost of DNA sequencing has been continuously decreasing, which made next generation sequencing, as an affordable tool in most laboratories. It has revolutionized the field of microbiology and microbial genomics. The microbial genome sequencing is now becoming a routine practice in microbiology and clinical microbiology. The third-generation sequencing has increased the possibility of having more of complete genomes than draft genomes. The contemporary era of single cell and metagenome-assembled genomes facilitate classification and functional annotation of unculturable microorganisms. Analysis of complete genome sequences is beginning to provide a great insight into many questions about the evolution of microbes and about microbial diversity, which is having profound effect on human health.

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Functional Genomics of Microbial Pathogens for Crop Improvement

9

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Abstract

Sequencing bacterial genomes over the past two decades has opened a new era in the analysis of pathogenic bacteria. Analyses of genomic sequences provided important insights into evolution of pathogenicity and antibiotic resistance. Genomic comparison between pathogenic bacteria and their less harmful relatives showed that virulence factors may be acquired by horizontal transfer of pathogenicity islands. Therefore, in addition to the presence of virulence and antivirulence genes, several features of the bacterial genome such as the GþC content, the genome size, and the proportion of genes encoding specific functions could help identify pathogens and assess their intrinsic virulence. Genomic information provides a background for additional high-throughput functional studies such as in silico metabolic modelling and wet-lab experimentation at the RNA and protein levels. Transcriptome studies of microbial pathogens using microarrays and RNA sequencing are particularly focused on changes in gene expression. Genome-wide targeted gene inactivation has been used to construct mutants and identify those with an altered phenotype. Targeted inactivation has been difficult to do in bacteria showing barriers to transformation. Although the role of bacteriophages in the evolution, virulence, and antibiotic resistance of bacteria has been recognized, only a few functional genomics studies of these

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viruses have been performed so far. Such studies are important for use of bacteriophages and their proteins in bacterial identification and infection control. It has been shown that several human diseases and disorders correlate with changes in microbiota profiles, suggesting that bacterial communities may play a causative role of disease. In conclusion, the expanding field of functional genomics provides powerful tools and insights for assessing the contribution of pathogens and microbial communities to disease as well as the characteristics of the host response and will contribute to development of new prevention and therapeutic strategies.

Keywords
Functional genomics · Microbial pathogens · Crop improvement

9.1 Introduction

Functional genomics is a new tool of cell biology that describes the functions and interactions of genes and proteins by making use of genome-wide approaches. High-throughput technologies, from studying individual genes and proteins and analyzing entire genomes and proteomes, have revolutionized in understanding the biology as by gaining the availability of huge information contained in the genomes of organisms. The functional genomics is an extreme complex and hefty approach to characterize the function of sequences; regulation and expression of individual genes at DNA, RNA, and protein level; their pathways; and finally entire genomes. It deals with the use of genome-wide approaches like genome and RNA sequencing for depicting the function and interaction of genes and proteins within an organism. To explore the gene product interactions and their influence on different phenotypical traits includes a systematic analysis of mRNA and protein expression to define gene functions. Information inferred from the various processes like coding and non-coding transcription, protein translation, and protein-DNA, protein-RNA, and protein-protein interactions, which are related to DNA sequence, gene expression, and protein function, are compiled together into numerous databases used to model interactive and dynamic networks that regulate gene expression, cell differentiation, and cell cycle progression (Muthamilarasan et al. 2013).

Over the past two decades, whole-genome sequences of microbes opened a new era in the analysis of genomic sequences, including pathogens and beneficial microbes which provided important insights into antibiotic resistance, phylogeny of pathogenicity, and virulence of important animal, plant, or human pathogens. Acquiring a better apprehension of the beneficial microbes living in, on, and around plants also plays a vital part in making up the nutrients that plants need in order to develop. High-throughput functional genomic technologies are accelerating progress in understanding the diversity of bacterial life and in developing a systems-level understanding of model bacterial organisms. Adoption of functional genomics approach will help in designing new and effective engineering techniques for crop

improvement and addressing food security, eventually. Whole genome sequencing of a number of important plant species started from *Arabidopsis thaliana* (2000), ranging from rice (2002) to chickpea (2013) and presently has reached till sequencing of diploid ancestors of peanut (2016), holds a pivotal role in accomplishing huge success in crop improvement programs observed in recent decades. Recent biotechnological advances are commoditizing with functional genomics platforms and enabling researchers to apply high throughput for genome studies to an increasing range of species. This means that in a relatively short period of time, these techniques, once limited to a handful of species though with complete genome sequences, will be used for studying a myriad of biological systems. There are many different techniques for utilizing functional genomics approach and in addition to that innovative technologies are accelerating the amount and complexity of data being collected.

Different approaches of functional genomics include:

1. *Transcriptomics*: studies of gene expression at the transcript or RNA level, including both mRNA and ncRNA gene expression in a cell.
2. *Proteomics*: approaches which focus on proteins being expressed in a biological system but also include study of protein structures.
3. *Metabolomics*: studies the metabolome, i.e., all metabolites in a biological system at a given time under a defined genetic background.
4. *Interactomics*: studies the molecular interactions between host and pathogen and encompasses such interactions. This branch has specific relevance to agriculture systems, under crop protection category, per se.
5. *Nutrigenomics*: studies effect of food and food constituents on gene expression. It focuses on identifying molecular level interaction between nutrients and other dietary bioactives with the genome.

Almost numerous varieties of powerful compounds can be inferred from microbial communities, as microbes are regarded as nature's primary chemists.

Microbes have played an important role in agriculture since the beginning of the agricultural practice itself. Microbes are an important component of the agricultural ecosystems. The crops and microbes have lived together on farms for millions of years. Traditionally, the native microbes have played both deleterious and beneficial roles in farming. While on one hand, the microbes afflict a number of diseases on crops causing significant yield losses, and on the other, they play myriad of beneficial roles in maintaining soil fertility and providing options for biological management of diseases and pests. Notwithstanding the beneficial or the destructive aspects of microbes in agriculture, one thing that clearly stands out is that crops and microbes are inseparable. Crop improvement through conventional plant breeding for over a century generally relied upon natural genetic variation present in the crop germplasm and to a large extent remained oblivious to the genetic resources of the diverse microbial communities. This was obvious as microbes could not be intermated with crops. Mostly, microbes were topically applied in agricultural practices

in the form of crude whole cell formulations or inoculants. The specific microbial genetic elements were first reported to be transferred to plants through human intervention using recombinant DNA and genetic engineering techniques in 1983 (Herrera-Estrella et al. 1983). Genetic engineering provided an opportunity to use specific microbial properties in a precise manner and built those properties into the crop genomes. Such transgenic crops containing microbial genes have been extensively cultivated by farmers in different parts of the world over the last two decades (James 2015). Microbial genes and regulatory genetic elements have been extensively used in crop improvement through genetic transformation. While a number of crop cultivars with microbial genes have already been released for cultivation, a significant number is under research and development.

Plant Pathogens and Diseases Are a Major Limitation to Crop Production Worldwide
Plant pathogens are a major restriction for crop production worldwide. The management of pathogens has mostly been achieved either using traditional breeding programs or through the use of pesticides, but nowadays, the genes derived from various microbes themselves have been used to combat microbial pathogens that cause many serious plant diseases. Plant defense responses are triggered by the recognition of pathogen-associated molecular patterns (PAMPs) that can potentially be any part of the pathogen including cell-wall proteins or flagella, toxins, etc. PAMPs are recognized by the plant using extracellular receptorlike kinases (RLK) that can perceive the PAMP and rapidly trigger a signaling cascade through MAP kinases resulting in basal immunity. Expressing multiple R-genes or R-genes in combination with other antimicrobial peptides has been shown to increase the effectiveness of the genes and is believed to increase the gene stability and decrease the potential for pathogens developing resistance. The use of transgenes that can modulate entire pathways through the use of master control switches such as NPR1 or those that work indirectly against the pathogens such as peroxidases would be much more difficult for pathogens to develop resistance toward and should be examined in more detail. Furthermore, the use of alternative promoters, which function in a tissue-specific manner or are induced during pathogen challenge as well as the beneficial gene of microbes has been used to virus-induced transient, Herbicide Resistant, Insect Resistant, Nutritional Improvement, Abiotic Stress Tolerance, microbial genes for hybrid seed production etc.

9.2 Microbial Genes for Pathogen Resistance

Microbial gene-based resistance has been achieved in the case of viral pathogens only, while such therapies are under research and development stage for various other pathogens, like fungi, bacteria, and nematodes. Virus-resistant transgenic crops were mainly developed by making use of gene silencing techniques such as RNAi and antisense RNA against viral genes (Ramesh et al. 2007). Different strategies employed for engineering virus resistance include expressing viral coat-protein

gene as transgene or expressing defective viral replicase or production of antiviral protein in transgenic plants or expressing antibodies specific to proteins involved in pathogenesis. Many virus-derived genes have been utilized to develop viral disease-resistant crops. The most successful example of the use of a microbial gene to develop virus resistance is that of papaya ringspot virus (PRSV)-resistant papaya (Gonsalves et al. 2004).

9.3 Virus-Induced Transient Gene Expression in Plants

Virus-induced gene silencing (VIGS) is mainly used for identification of gene function. This technique utilizes viral vectors that carry gene fragment of the target gene. As a result of virus induction, dsRNA molecule is produced, and this leads to starting of RNA-mediated gene silencing. VIGS is considered as a reverse genetic tool that provides an alternative way for characterization of gene functions in a transient way. In this part, mechanisms, development, and improvement of this method have been examined.

9.3.1 Basic Mechanism of VIGS

VIGS technique actually uses antiviral defense mechanism of plants in which post-transcriptional gene silencing (PTGS) occurs (Baulcombe 1999a). In normal conditions, when plants are infected with unmodified viruses, viral genome is targeted and destroyed. If the virus vectors carry a part of gene of interest, the corresponding mRNA of targeted gene is cleavage (Lu et al. 2003). VIGS term was firstly used by A. van Kammen who indicated resistance against viral infection in plants (van Kammen 1997). Basically, this technique is based on silencing of gene of interest (GOI) through RNA-mediated defense system in plants. The main idea of VIGS includes transferring of viral RNA or DNA that also involves a small portion of specific gene sequence into plants (Baulcombe 1999b). The target gene sequences are firstly inserted into viral genome without any disruption in its infectivity (Lu et al. 2003). So, this method carries an advantage that enables knocking out of a specific gene without affecting other genes in plant genome (Unver and Budak, 2009). In VIGS method, RNA-induced gene silencing mechanism occurs in which 21–25 nucleotide sequences of small interfering RNAs (siRNAs) are produced and directed to specific regions of target mRNAs for cleavage. This process takes place at posttranscriptional level (Klahre et al. 2002). From the long double-stranded RNAs (dsRNA), siRNAs are processed with RNAse-like enzyme known as DICER. At the end, siRNAs are connected to RNA-induced silencing complex known as a RISC (Unver and Budak 2009). RISC containing siRNA has an ability to bind target mRNA region in which complementary with the specific siRNA is found. So, sense strand from target gene mRNA and antisense strand from siRNA are combined with each other, and specific regions of mRNA targets are degraded.

This is the general mechanism for siRNA degradation process. Although all main steps are similar with siRNA degradation process, there are some details for VIGS method in plants for silencing of GOI. After the modification of viral genome that is joined with target gene portion, this construct is transformed into plants using *A. tumefaciens*. In the plant cell, foreign RNA molecule is transcribed and replicated by an endogenous RNA-dependent RNA polymerase (RDRP) enzyme. As a result, long dsRNA molecule is produced and then recognized by DICER. After the cleave of dsRNA into siRNAs, they are noted by RISC complex and converted them to single-stranded siRNAs. The RISC complex utilizes these single-stranded siRNAs to find out their complementary sequences in RNA pool of the cell. After finding right sequences, they are degraded (Ding and Voinnet 2007).

9.3.2 Methodology Development for VIGS

There are different types of viruses that are well adopted as VIGS vectors for silencing of target gene in plants. Both RNA tobacco mosaic virus, TMV (Kumagai et al. 1995); potato virus X, PVX (Faivre-Rampant et al. 2004); barley stripe mosaic virus, BSMV (Holzberg et al. 2002); bean pod mottle virus, BPMV (Zhang and Ghabrial 2006); pea early browning virus, PEBV (Constantin et al. 2004); tomato bushy stunt virus, TBSV (Hou and Qiu 2003); African cassava mosaic virus, ACMV (Fofana et al. 2004); and tomato yellow leaf curl China virus, TYLCV (Tao and Zhou 2004), are used for VIGS applications in different silencing host plants. Among them, *Nicotiana benthamiana*, *N. tabacum*, *Solanum lycopersicum*, *A. thaliana*, *Capsicum annuum*, *Opium poppy*, *Aquilegia vulgaris*, *Hordeum vulgaris*, *Glycine max*, *Pisum sativum*, *Medicago truncatula*, *Lathyrus odoratus*, *Populus trichocarpa*, *Oryza sativa*, *Zea mays*, *Manihot esculenta*, and *Lycopersicon esculentum* are widely used for host plant species in different gene silencing studies. Using VIGS method, there are many gene characterization studies such as different plant development stages (Senthil-Kumar et al. 2008), disease (van der Linde et al. 2011), nematode (Mao et al. 2011) and insect resistance (Mantelin et al. 2011), and abiotic stress (George et al. 2010).

The first VIGS application was performed with TMV that caused knockdown of *pds* gene in *N. benthamiana* (Kumagai et al. 1995). It was shown that the minimum sequence length of RNA for gene silencing was detected in different studies. They indicated that 23-nucleotide RNA was the minimum for 100% homology to the target gene. However, longer similar sequences were required for efficient PTGS (Thomas et al. 2001). Modified TRV is another VIGS vector that has been used for more than 15 years for gene silencing in plants. The main benefits of TRV vector are easy transfer into plants, especially Solanaceae family members, and higher spreading capability throughout whole plant parts (Unver and Budak 2009). Using this vector, gene silencing was succeeded in *N. benthamiana* (Liu et al. 2002a) and tomato (Liu et al. 2002b). Traditionally, VIGS vector is located between right and left borders of TDNA (Liu et al. 2002b). Strong promoters such as 35S or duplicate

35S promoters and terminator such as a ribozyme were added to cassette and inserted into *A. tumefaciens*. These regulators provide more effective and faster spreading of TRV vectors. pYL156 and pYL279 were TRV vectors with double 35S promoters that caused infection of different plant species (Liu et al. 2002a). PVX is an RNA virus that has a limited host range when compared with TMV-based vectors.

9.3.3 Recent Improvements of VIGS

VIGS is a widely used genetic tool for plant functional genomics. VIGS application in plants takes place for a short duration of time that takes approximately only few weeks throughout plant life cycle. This provides some advantages for researchers who study with plant genomics. Firstly, VIGS is an easy, fast, and cheap technique for gene silencing in plants. Secondly, there is no requirement for stable plant transformation. Thirdly, there is no need to know the whole sequence of a gene that is silenced. In other words, only having small portion of interested gene sequence is enough for VIGS utilization. In addition, this technique can be used for both forward and reverse genetics. In polyploidy plants such as wheat and cotton, some genes have multiple copies or belong to multiple family members. Another advantage is that such genes are also silenced. Lastly, functional analysis of genes whose mutation causes lethality in sexually propagated plants can be examined. Because of these advantages of VIGS, this method has been applied to different kinds of plants including monocots and dicots. However, researches have still worked on VIGS to improve this technique.

If the VIGS covered the entire life cycle of a plant, it is known as long-duration VIGS, which is replaced with mutants or stable RNA interference techniques. Especially, long-duration VIGS is practical for abiotic and biotic stress studies (Senthil-Kumar and Mysore 2011a). When stress tolerance is evaluated, stress can be applied to plant from the beginning of the seedling to the terminal growth stage. For understanding the function of genes, long-duration VIGS enables opportunity to survive for many years or until the death of the plant (Senthil-Kumar and Mysore 2011b). The major drawback of VIGS is that this method does not change the genome of target plants, unlike stable RNAi and mutant plants. However, different studies indicated that gene silencing using VIGS method was achieved with heritable manner. Barley stripe mosaic virus (BSMV)-mediated VIGS provided an opportunity to transfer gene silencing process for up to six generations in barley and oat, rice, and purple false brome. Apple latent spherical virus (ASLV)-mediated VIGS has a higher silencing efficiency potential than BSMV-mediated VIGS. It is shown that ASLV-mediated VIGS in 15 different plant species including Brassicaceae, Leguminosae, Cucurbitaceae, and Solanaceae families has been shown to be transmitted progeny (Igarashi et al. 2009). TRV-mediated VIGS is the most selected VIGS method for a wide range of plant species especially dicot plants. Its application in *N. benthamiana* and tomato

showed that gene silencing has been transferred to progeny (Senthil-Kumar and Mysore 2011b). These types of vectors cause non-integration-based transmissible PTGS that provides transmission of progeny through the next generation (Senthil-Kumar and Mysore 2011a). When compared with short-duration VIGS, it has some advantages. For example, the vectors including TRV (Senthil-Kumar and Mysore 2011b) and BSMV have mild viral symptoms in the progeny. Another advantage is that gene silencing occurs during seed dormancy, seed germination, and seedling emergence. Hence, perfect silencing can be accomplished in whole parts of plant including root, stem, and cotyledons. Another type of VIGS system is transgene-free stable (TGS). In contrast to non-integration-based transmissible PTGS, virus vectors in TGS can bring about DNA methylation that occurs on promoters of target gene and is performed by siRNA (Kim and Kim 2011). In a recent study, a cucumber mosaic virus (CMV)-based vector has been used for gene silencing. Promoter of interested gene has been targeted for methylation in petunia and tomato (Sonoda and Nishiguchi 2000). This VIGS system is considered as a nontransgenic approach because there is no integration of viral insert into plant genome.

9.4 Herbicide-Resistant Transgenic Plants

The use of genetic engineering techniques to develop glyphosate-resistant (GR) crops was a scientific discovery that leads to revolutionizing the weed management strategies (Green 2012). Glyphosate (*N*-(27)-glycine) is a powerful and most widely used broad-spectrum herbicide targeting the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. Up till now, many genes have been shown to provide a glyphosate resistance effect to different cells (Yu et al. 2015). By using these genes, various herbicide-tolerant transgenic plants have been generated. Five different microbial genes have been used to develop glyphosate-tolerant transgenic crops. Out of five, two genes, namely, *cp4 epsps* from *A. tumefaciens* strain CP4 and *ep-sps grg23* from soil bacterium *Arthrobacter globiformis*, encode for a glyphosate-insensitive 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme. EPSPS, the target of glyphosate, is a shikimate pathway enzyme that is involved in the biosynthesis of essential aromatic amino acids in plants (Dill et al. 2008). The remaining three microbial transgenes, *gat 4601* and *gat 4621* from *B. licheniformis* and goxv*247* from *Ochrobactrum anthropi* strain LBAA, encode enzymes that detoxify glyphosate to nontoxic by-products. The *A. tumefaciens* strain CP4, source of *cp4 epsps*, was isolated from a waste stream in a glyphosate-manufacturing facility (Barry et al. 1992). Since weed control with classical tools was time-consuming and costly, the usage of GR crops made weed management easy, efficient, economical, and environmentally compatible. The first herbicide-resistant transgenic plant was produced in 1986 by introducing EPSPS gene into soybean cells (Shah et al. 1986).

9.5 Insect-Resistant Transgenic Plants

One of the most important abiotic stress factors reducing agricultural productivity is pests. So, the second important trait introduced by *Agrobacterium*-mediated gene transfer into plant cell was insect resistance. There are two main approaches for the production of genetically engineered insect-resistant plants. In the context of the first approach, insect-resistant transgenic plants are generally obtained through the transferring of genes encoding crystal toxin proteins (Cry proteins) from *Bacillus thuringiensis*. These proteins inactivate their targets through the affecting guts. *Cry* genes code resistance in plants against a variety of insects belonging to Lepidoptera (Zhao et al. 2014), Coleoptera (Tohidfar et al. 2013), Hemiptera (Rausch et al. 2016), and Diptera (Andrews et al. 1987). The first example of insect-resistant transgenic plant was transgenic tobacco plant produced through the introduction of *bt* genes by using *A. tumefaciens* (Hilder et al. 1987), although the first commercially available bt transgenic plant, a transgenic maize generated for controlling corn borer (*Ostrinia nubilalis*), was produced using biolistic method. The number of bt transgenic species produced via *Agrobacterium*-mediated gene transfer has dramatically increased. GM crops with *bt* genes were globally planted over 35 million hectares in 13 different countries in 2014, and they constitute 15% of all GM crops (James 2015). Several strategies of biological control of insects have been reported using different microorganisms such as fungi, bacteria, nematodes, and viruses (Mazid et al. 2011). The first insect-resistant transgenic plant containing microbial gene was produced some 20 years ago. Since then, a huge number of novel resistance genes of different microbial origins were discovered and used for plant transformation. Different *cry (Bt)* genes from the soil bacterium *Bacillus thuringiensis* have been extensively used to develop insect resistance in crop plants. In the middle of the 1990s, maize and later cotton with *cry* genes were released for cultivation. Till date, over 350 *B. thuringiensis* endotoxins have been identified (Crickmore 2006). *B. thuringiensis* is a gram-positive bacterium producing insecticidal protein crystals during sporulation (Williams et al. 1992). There is a large family of delta-endotoxins classified as Cry I, II, III, IV, V, etc., depending on molecular relatedness and activity against insect larvae. Insect larvae feed on foliage and ingest the toxin; after ingestion, the toxin binds to specific receptors in the gut and is solubilized and activated by proteinases in the insect midgut epithelium. The activated toxins induce perforation in the midgut epithelial membrane that results in cell lysis and leads to death of the larva.

9.6 Nutritional Improvement

After the successful use of recombinant DNA techniques in the development of plants, improving the nutritional quality of food crops has become an important target. Genes from different sources were transferred into plant cell to change the nutritional content of plants. For instance, *phytoene synthase* (*psy*) and *lycopene β-cyclase* (*β-lcy*) genes from *Narcissus pseudonarcissus* were transferred into rice

genome by using *Agrobacterium*-mediated gene transfer to increase vitamin A content (Beyer et al. 2002). Likewise, genetic engineering of soybean plant was carried out with *A. tumefaciens* including RNAi construct for β-subunit gene in 7S globulin protein to decrease antinutritional effects (Qu et al. 2016). Other good examples for improved nutritional value of crops were transgenic soybean and maize with an increased content of β-carotene and lysine, respectively (Kim et al. 2012). The seeds of transgenic soybean plants had ~62-fold higher β-carotene than nontransgenic seeds (Kim et al. 2012).

9.7 Abiotic Stress Tolerance

Drought, salinity, and cold are the most important environmental stresses decreasing the agricultural production in all over the world. To increase the productivity, it is necessary to increase tolerance against the environmental stresses. To generate abiotic stress-tolerant crops, a combinatorial approach consisting of plant biotechnology, genetics, and breeding is required. Plants can be genetically engineered to improve abiotic stress tolerance. Many organisms have special genes that the expression pattern significantly changed during the environmental stresses. Some of these genes have been cloned and transformed into plants by using *Agrobacterium*-mediated gene transfer. A drought-tolerant maize cultivar expressing a cold-shock protein (*cspB*) gene from the soil bacteria *B. subtilis* has been released. The *cspB* gene helps the plants to cope with drought stress by stabilizing the cellular RNA (Castiglioni et al. 2008). The field trial results indicated an average increase of five bushels of maize per acre during drought as compared with other competitive drought-tolerant hybrids that do not contain this microbial gene (Waltz 2014). Besides maize, a drought-tolerant sugarcane cultivar containing microbial gene, *betA*, has been released (Waltz 2014). The two versions of *betA* gene isolated from *E. coli* or *Rhizobium meliloti* encode choline dehydrogenase. Choline dehydrogenase converts choline into betaine aldehyde, which is then converted to osmoprotectant compound glycine betaine by enzyme betaine aldehyde dehydrogenase. The glycine betaine osmoprotectant acclimatizes plants to abiotic stress conditions such as drought, salinity, and low temperature by stabilizing the macromolecules and maintaining integrity of plasma membrane. This sugarcane produces 20–30% more sugar during drought as compared with the conventional counterpart (Waltz 2014).

9.8 Microbial Genes for Hybrid Seed Production

Hybrids are known for higher yield, as they exhibit enhanced performance than their parental lines, a phenomenon known as heterosis or hybrid vigor. The generation of male sterile plants hastens hybrid seed production (Greenland et al. 1997). Male sterile transgenic plants can be generated by overexpressing transgene that disrupts or interferes with the development of stamens or the tapetal cells of anther. There are many genes from different microbial sources that can disrupt pollen or

anther development and hence lead to male sterile transgenic plants. The *barnase/barstar* system has been exploited to a great extent. In *barnase/barstar* system, male sterile lines are obtained by expressing *barnase* gene under a tapetum-specific promoter TA29 in transgenic plants. *Bacillus amyloliquefaciens* bacteria are the source of *barnase* gene that encodes for an RNase (ribonuclease) enzyme. This RNase is lethal for cells, and specific expression in the tapetum layer kills tapetum cells of anther, while it has no effect on other cells or tissues; hence, sterile pollens are produced by transgenic plants (Mariani et al. 1990). *Barnase* gene is linked with *bar* gene (glufosinate herbicide-tolerant gene from *Streptomyces hygroscopicus* for easy selection of transgenic plants by spraying herbicides. *Barnase* strategy has been used by many workers for the production of transgenic male sterile plants in various crops (Banga et al. 2006). The restorer lines can be obtained by tapetum-specific expressions of another *B. amyloliquefaciens*-derived gene, *barstar*, which encodes ribonuclease (RNase) inhibitor. These restorer lines are used as male parent, while male sterile lines are used as female parent in hybrid seed production program (Mariani et al. 1992). The F_1 hybrids contain both *barnase* and *barstar* genes, and they produce fertile pollens.

9.9 PGPR-Mediated Nitrogen (N) Fixation

All organisms require N for the synthesis of several biomolecules like proteins and nucleic acids. However, the main source of N in nature is the atmospheric nitrogen (N_2). Plants are incapable to use directly the available atmospheric N (80%) due to the stable nature of molecular nitrogen (N_2). BNF is responsible for the reduction of N_2 to ammonia (NH_3). The fixation of atmospheric N is catalyzed by nitrogenase enzyme complex, a highly conserved enzyme that comprises two metalloproteins, FeMo protein and Fe protein, present in the N-fixing bacteria majorly rhizobacteria. Among the rhizobacteria, rhizobia are the group of bacteria that have the ability to fix the nitrogen by forming nodules in leguminous plant and convert it into ammonia and make it available to the plants (Ahemad and Kibret 2014). Main examples of symbionts are *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Mesorhizobium* with leguminous plants and *Frankia*, with nonleguminous trees and shrubs. These rhizobacteria mainly use two types of mechanism for fixing the atmospheric nitrogen. In symbiotic nitrogen fixation, both bacteria and plant live in mutualistic relationship. Bacteria first enter into the root and form nodules, which is the region where nitrogen fixation takes place.

On the other hand, nonsymbiotic nitrogen fixation is carried out by free-living diazotrophs that are able to stimulate nonlegume plant growth such as in radish and rice plants. The genera that mainly belong to nonsymbiotic nitrogen-fixing rhizospheric bacteria are *Azoarcus*, *Azotobacter*, *Acetobacter*, *Azospirillum*, *Burkholderia*, *Diazotrophicus*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas*, and *Cyanobacteria* (*Anabaena* and *Nostoc*) (Bhattacharyya and Jha 2012). The genes responsible for nitrogen fixation, called *nif* genes, are present in both symbiotic and free-living systems. *Nif*-related structural genes encode the enzyme nitrogenase that

converts atmospheric nitrogen to other nitrogen forms such as ammonia. This is done via sequential factors like activating Fe protein, iron-molybdenum cofactor biosynthesis, and electron donation and upregulating other regulatory genes required for the synthesis and function of the enzyme. Inoculation by biological nitrogen-fixing plant growth-promoting rhizobacteria on crop provides an integrated approach for disease management and growth-promotion activity and maintains the nitrogen level in agricultural soil.

9.10 Basic Mechanism of Genome Editing

Targeted alterations of any gene or genome in the living cells or organisms require a powerful tool and well-characterized mechanism. Basic mechanism of GE and its successful implementation involve designing and construction of nucleases in the form of DNA plasmid or mRNA, selection of suitable host, identification and binding of target DNA sequence, cleavage as single or double DSB at the desired site, incorporation of desired sequence by HDR or random insertion or deletion by NHEJ, screening of desired change by DNA sequencing, and phenotype analysis (Maeder and Gersbach 2016). Specific designing and engineering of each type of nucleases are discussed later. Each technique of GE follows distinct process of recognition and binding of target DNA sequence. Delivery of engineered nucleases containing DNA constructs is achieved by *Agrobacterium*-mediated delivery and polyethylene glycol delivery into protoplasts, through nonintegrating viruses and microparticle bombardment method.

9.11 Microbial Genome and Plant Microbe Interaction

The first batch of genome sequences of plant pathogenic fungi and oomycetes marked the emergence of a new research field centered on the genome biology of these important pathogens. The genome sequences have revealed a lot of new information about the evolution of these fascinating microorganisms and the genomic features that underlie their success. Most strikingly, several lineages of filamentous plant pathogens, particularly the biotrophs, are remarkable among pathogenic organisms in displaying an evolutionary trend toward bigger, transposable element-rich genomes (Raffaele and Kamoun 2012). Now, the genome-based studies on plant-associated microorganisms have changed our understanding of plant pathogens and also transformed our knowledge of mutualistic and commensal interactions with economically important plants (Guttman et al. 2014).

In filamentous fungi, the effector genes are also commonly found in association with rapidly evolving segments of the genome, such as repeat-rich regions, or on accessory chromosomes (ACs). For example, *AvrPita* in *M. oryzae*, *SIX* genes in *F. oxysporum*, and the PEP cluster in *Nectria haematococca* are all located on ACs. *Zymoseptoria tritici* has several ACs that are well described, though, unlike other fungal pathogens, they have never been associated with pathogenicity (Croll et al.

2013). Other well-characterized necrotrophic effectors, such as ToxA in *Pyrenophora tritici-repentis* and Tox3 and Tox1 in *Parastagonospora nodorum*, were successfully identified using culture filtrates that induced necrosis when infiltrated into susceptible wheat varieties (Liu et al. 2012). This approach has recently identified two necrosis-inducing proteins, ZtNIP1 and ZtNIP2, in *Z. tritici*. The heterologous expression and infiltration of these proteins into wheat also revealed cultivar specificity. Recently, Solomon and coworkers have developed a gene tree sorting method that quickly identifies groups of isolates within a single-gene alignment whose sequence haplotypes correspond with virulence scores on a single wheat cultivar (*Z. tritici*) (McDonald et al. 2016). Using this method, they have identified 100 candidate effector genes whose gene sequence correlates with virulence toward a wheat cultivar carrying a major resistance gene.

9.12 Genome Evolution in Bacterial and Fungal Plant Pathogen

Pathogenic fungi and bacteria can lead to severe economic losses due to infected crops; therefore, it is of great concern to food security. The increasing global transportation of plant and plant products creates new combinations of their associated pathogens. Such events need serious attention because they may lead to the emergence of diseases with new epidemiological properties or host specificities (Brasier 2001). Further, hybridization events have also given rise to a variety of genomic constitutions and evolutionary consequences. Compared with animals and plants, the genome of fungi exhibits gene-dense genomes, with an average estimated size of ~ 37 Mb and ranging between 6.5 Mb for *Pneumocystis carinii* and 795 Mb for *Scutellospora castanea* (Gregory et al. 2007). There is also significant variation in chromosome numbers in fungi, with the smallest number of 3 in the ascomycete *Schizosaccharomyces pombe* and the largest number of 20 in the basidiomycete *Ustilago hordei* and the chytrid *Batrachochytrium dendrobatidis* (Gregory et al. 2007).

Genome evolution has taken place mainly by three main forces, that is, gene gain, gene loss, and gene change. Comparative genomics showed that fungi and bacteria have different modes of host adaptation on the genomic level. The pathogenic lifestyle of fungi suggests the tendency for reduced genome size in fungi (Yuen et al. 2003). This signature of adaptation can be acquired by losing either genes or whole metabolic pathways that are no longer necessary; for example, *Hemiascomycetes* have lost the genes needed to survive on the carbon source galactose that was irrelevant within a new host environment (Hittinger et al. 2004). In spite of common themes in fungal evolution, fungi are strikingly diverse at the genome level and mostly showing lineage-specific evolution. They not only are highly divergent in DNA sequences but also are striking changes in the order and localization of homologous genes among genomes. For example, the comparison of ascomycetes *Neurospora crassa* and *Magnaporthe grisea* reveals that their genomes have only 74% identity at the amino acid level and with virtually no similarity

between the chromosomal fragments (Dean et al. 2005). Furthermore, most pathogenic fungi have also experienced the expansion of specific gene families related to functions that facilitate the infection of the host. An example of how the expansion of specific gene families provides pathogenic potential to an organism is given by the genome of *Penicillium marneffei*, the only known pathogenic fungus of the *Penicillium* genus. Compared with its progenitors and relatives, *P. marneffei* has adopted reductive genome evolution (17 Mb compared with ~30 Mb in other *Penicillium* species), and its genome is rich in secondary metabolite genes and thioester-mediated nonribosomal protein synthesis (Yuen et al. 2003).

The genomic organization of a symbiotic fungal species can now be studied with the increase in genome-sequence projects and the availability of the genome sequences of the several fungal species (Sharma, 2016). One of the earlier-sequenced basidiomycetous fungi, *Laccaria bicolor*, has a genome of 65 million base pairs and 20,000 predicted genes, which is relatively larger than other fungi (Martin et al. 2008). Only 70% of the predicted genes have homologues in other fungi, and their size can be partly accounted for by a large number of transposons and repeated sequences and by the presence of large lineage-specific multigene families. An earlier report provides the evidences for the expansion of numerous protein gene families related to the functions that make possible the symbiotic relationship between *L. bicolor* and its host *Populus trichocarpa*. In contrast, the genome of *L. bicolor* shows a marked reduction in the gene families coding for plant cell-wall degradation enzymes, while these families are well represented in the genomes of many other fungal pathogens (Martin et al. 2008).

Further, in many fungal pathogens, genetic variations created by chromosomal rearrangements have been reported to favor adaptation to novel hosts or nutritional environments (Larriba 2004). For example, in the pathogenic yeast *Candida albicans*, phenotypic mutants derived in vitro often exhibit altered karyotypes and mutation frequencies varying between 10^{-5} and 10^{-2}, depending upon the strain (Rustchenko 2007), whereas, in *Fusarium graminearum*, the localized and highly polymorphic genomic regions are significantly enriched with genes favoring plant infection, such as secreted proteins, major facilitator transporters, and cytochrome P450s (Cuomo et al. 2007). Phytopathogenic bacteria are a group of bacteria pathogenic to plants and therefore generate large implications on agriculture and food security. Those bacteria are regarded as equally important to agriculture as viral (Scholthof et al. 2011) and fungal pathogens (Dean et al. 2012). Analyses of genome sequences in bacteria have demonstrated that many of the genes required for virulence are restricted to pathogenic organisms and that they have been introduced into the genomes by horizontal gene transfer. Horizontal gene transfer, the nonsexual transfer of genetic material between organisms, is well established as a major evolutionary process in bacteria, for example, bacterial pathogens to acquire new virulence functions. Genes on plasmids or secondary chromosomes have been shown to evolve faster, and thus, together with the capability of exchange, plasmids can represent a hot spot of evolution for phytopathogenic bacteria.

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10 Role of Microbial Genomics in Plant Health Protection and Soil Health Maintenance

Arpna Ratnakar and Shikha

Abstract

Global increase in agricultural production from a gradually decreasing and degrading land resource has placed immense pressure on the agroecosystems. Soil microbial populations are engaged in a web of interactions affecting plant fitness as well as soil quality. They are engaged in core activities ensuring the productivity as well as stability encompassing agricultural systems and natural ecosystems.

Agricultural sustainability can be improved through optimal use and management of soil fertility along with physical properties, which altogether depends upon soil biological processes and biodiversity. Soil fertility in addition to other properties, e.g., texture, aeration, available moisture, etc., known to support agricultural production has been found to depend on the biomass, metabolites, and activities of microorganisms. Hence, an understanding of microbial diversity perspectives in agricultural scenario is not only important but also useful to land upon measures which may perform as indicators of soil quality and plant productivity.

Soil microbial community structure consists of two main drivers, viz., plant type and soil type. At times the soil, while in others the plant type, happens to be the key factor determining soil microbial diversity which is intricately related to the microbial interactions in soil, interactions between microorganisms and soil in addition to microorganisms and plants. Soil microorganisms mediate the biogeochemical cycling of carbon, nutrients, and trace elements by catalyzing redox reactions which moderate atmospheric composition, water chemistry, and the bioavailability of elements in soil.

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Positive plant-microbe interactions in the rhizosphere are the core determinants of plant health and soil fertility. Plants provide specific habitats to the microbial communities, broadly categorized under the rhizosphere, phyllosphere, and endosphere. A symbiotic relationship exists between plants and associated microorganisms as well as high structural and functional diversity within plant microbiomes. Plant-associated microbes interact with their host in essential functional contexts. They can stimulate germination and growth, help plants to disease resistance, promote stress resistance, and influence plant fitness.

Keywords
Microbes · Soil · Plant · Restoration · AM

10.1 Introduction

Soil life diversity is predominantly microbial, and there remains a lot that is not understandable as yet, e.g., the structure and function of the soil microbiome (Little et al. 2008) and the entire diversity of soil microorganisms, in addition to their genetic capacity. A very intensive and significant interaction exists in the environment between the soil, plant, and microfauna.

Rhizosphere represents the roots of plants along with the surrounding soil wherein the biochemistry of soil is maximally influenced by root. Rhizospheric microorganisms tend to compete for water, nutrients, and shelter and at times improve their competitiveness through symbiotic association with plants (Hartmann et al. 2009). Such associations play significant roles in the ecological growth and fitness of their counter host. Hence, an understanding of the rhizospheric microbial ecology, along with the microbial diversity residing in the rhizosphere, is important prior to application of soil microbial technology within the rhizosphere. Microbial rhizospheric inhabitants include fungi, bacteria, algae, and actinomycetes. According to an estimate, 20–40% of carbon fixed photosynthetically gets transferred to the rhizosphere, thereby feeding the microbial community besides influencing their activities and their composition (Bais et al. 2004). A 10- to 100-fold variation in microbial population exists in the rhizosphere as compared to soil (Campbell and Greaves 1990). Microorganisms and their products are known to influence the roots of plants negatively, positively, as well as neutrally (Broeckling et al. 2008). Overall, the rhizosphere plays a significant role in maintaining plant growth and productivity through variety of interactions and communications among the root and microorganisms.

Plant-microbe interactions are greatly influenced by the surrounding environment in which they live (Drakare 2002; Tetard-Jones et al. 2007). Nutrient allocation, plant productivity, and histological chemistry may remarkably vary depending

on the identity and presence of adjacent individuals (Gersani et al. 2001; Murphy and Dudley 2009; Broz et al. 2010). Effects of a specific plant on the soil microbiome seem to be mediated by the community related to that plant (Bakker et al. 2013a, b; Schlatter et al. 2015). Soil microbial communities significantly give a feedback of the plant fitness through the suppression of plant pathogens. *Streptomyces* have long been used as inoculative biocontrol agents for plant pathogens (Yuan and Crawford 1995; Liu et al. 1996; Xiao et al. 2002).

The sole objective of sustainable agroecosystems is to oversee soil fertility which is a basic resource of agricultural production. Evaluation of soil fertility includes a keen observation of the properties of soil like physical, chemical, and biological changes. Soil enzyme activity gets influenced by several factors, viz.. natural parameters (e.g., geographic location, seasonal changes, physicochemical properties, in situ distribution, content of organic matter and clay, etc.). These parameters perturb enzyme activity level by influencing both the enzyme production by organisms as well as plants and their persistence under natural conditions. Among several biological features, the most frequently used index of soil fertility happens to be soil enzymes because they are extremely sensitive and respond quickly to alterations in soil management compared to other soil variables. Soil enzymes are sensitive indicators of soil fertility due to their ability to catalyze the key biochemical reactions such as nutrient cycles in soil, besides being sensitive and responsive to changes caused by either natural or anthropogenic factors easily, and can be measured easily, the process being less time-consuming, hence allowing analysis of large number of sample in small amount of soil.

The physicochemical properties of soil affect the stabilization as well as immobilization processes of most of the extracellular enzymes (Gianfreda and Bollag 1996). Anthropogenic agricultural practices and environmental pollution (e.g., heavy metals, pesticides, tillage, fertilizers, PAHs) affect the structural and chemical characteristics of the soil which not only affect the biological composition and diversity of microorganisms but their metabolic activities as well, leading to either enhancement or suppression of enzyme production modulating the overall activity of enzymes in soil (Gianfreda and Bollag 1996). Soil enzymes are indispensable in soil functioning; i.e., they play an important role in transformation and decomposition of organic materials, ensuring availability of nutrients to plants, affecting N_2 fixation, nitrification, and denitrification processes, detoxification of xenobiotics, such as industrial wastes and pesticides, etc. (Dick 1997).

Plants and microbes together have evolved intimate relationships which enable them to coexist (Nihorimbere et al. 2011). Identification of microorganisms from the plant microbiome that can be exploited for improving plant growth and health is a difficult task. In order to enhance plant growth and health, it becomes essential to identify the type of microorganism present in the rhizosphere microbiome and their respective functions. In this chapter, an attempt has been made to review the main functions of rhizospheric microorganisms with reference to genomics and the various impacts on plant health.

10.2 Role of Microbial Genomics in Plant Health Protection

10.2.1 Plant Growth-Promoting Rhizobacteria (PGPR) in Agriculture and the Environment

PGPR includes genera such as *Pseudomonas* and *Bacillus* species which are naturally rhizosphere-inhabiting bacteria. Canola, *Arabidopsis*, barley, rice, and bean are some plant species from which these microorganisms have been isolated (Persello-Cartieaux et al. 2003). PGPRs are applied as bioinoculants for phytostimulation, for biofertilization, and for biocontrol. Contribution of PGPR may be exerted through various ways, viz., an increased growth and productivity of plants, increased shoot growth due to production of phytohormones such as auxins and cytokinins, etc.

PGPRs can induce defense programs likes systemic acquired resistance (SAR) and induced systemic resistance (IST), thereby reducing phytotoxic microbial communities (Mantelin and Touraine 2004; Yang et al. 2009). *Pseudomonas* is a genus comprising omnipresent gram-negative bacteria inhabiting diverse environmental niches such as the rhizosphere and rhizoplane. Some *Pseudomonas* species behave like plant pathogens antagonizing with plant-beneficial bacteria such as *P. fluorescens*, *P. putida*, *P. aureofaciens*, and *P. chlororaphis* (Venturi 2006) and lead to the manifestation of traits which directly influence growth and plant disease resistance.

10.2.2 Plant Nutrients and Relationships with Other Physiological Processes

Mineral elements play an important role in plant physiology. Plant produces organic matter from mineral elements present in soil and natural environment. Deficiency of at least one of these essential elements may cause physiological disorders, like cell death (Koshiba et al. 2009).

For an increase in agricultural food production, application of fertilizers containing various nutrients, including N, P, and, K, is required. The present availability of plant genome sequences and the advancements in molecular biology techniques have increased the identification of the assimilation pathways, the genes responsible therein, as well as the nutrients present.

Rock phosphate, a source of fertilizer, is a limited nonrenewable resource. Microorganisms can use rock phosphate and make them available to plants. Some plant genomes offer new study and research application on symbiotic interactions toward plant growth regulation as well as efficient use of fertilizer inputs, e.g., of the plants including *Lotus japonicus* (Sato et al. 2008) and *Medicago truncatula* (Li et al. 2012), along with symbiotic microorganisms such as *Rhizobium* spp. (Servin-Garciduenas et al. 2012).

In the aforesaid context, an attempt has been made to discuss some important advances dealing with the metabolism of the major macronutrients, namely, N, P,

and K, on the basis of both genomic and biochemical studies, together with microbially assisted plant nutrient transformations.

10.2.2.1 Nitrogen

Plants take nitrogen (N) in the form of nitrate (NO^{3-}) and ammonium (NH^{4+}), and in legumes N can be absorbed through symbiotic nitrogen fixation processes. This process is driven by bacteria, the sole organisms having the key enzyme nitrogenase, which catalyzes the reduction of atmospheric N_2 to ammonia through symbiotic root nodules (Leigh 2002). For sustainable agriculture there is a need for efficient utilization of nitrogen sources and an overall balance between them, but some species exhibit a strong preference for one ionic species over the other (Wang et al. 2014). Recent advancements in the field of biofertilizers have shown that it is possible to exploit N-fixing bacteria efficiently without an application of excessive concentrations of N-rich mineral fertilizers; genera suitable for this include N-fixing bacteria *Rhizobium* and free-living rhizobacteria of the genera *Azospirillum*, *Azotobacter*, *Enterobacter*, *Pseudomonas*, *Bacillus*, *Serratia*, and *Streptomyces* (Reddy 2014). It has been reported that plant growth promotion can be achieved through microbial mobilization of various nitrogen sources, as revealed by higher yield in plants inoculated with bacterial strains (Shaharoona et al. 2008; Adesemoye et al. 2009), although, the source of N was found to be derived directly from the ammonium sulfate fertilizer compared to organically bound soil N (Adesemoye et al. 2010).

10.2.2.2 Phosphorus

It is well documented that phosphate (P) is adsorbed by either Ca or Mg in basic soils or by Fe and Al in acid soils getting precipitated as orthophosphate (Vance 2001; Lopez-Arredondo et al. 2014). P-solubilizing bacteria play a significant role in availability of phosphate nutrition to plants through release of P from inorganic and organic soils (Mohammadi 2012). Microorganisms accomplish phosphate solubilization by lowering the pH and P mineralization through production of organic acids and acid phosphatase enzymes. P-solubilizing bacterial inoculants increase uptake of P from the soil, enabling the use of rock phosphate for crop production (Nobandegani et al. 2015). Singh and Satyanarayana (2011) elaborated the importance of phytases and the microorganisms involved in their production, in the use of organic phytate sources. Most of the P-solubilizing microorganisms are bacterial strains belonging to genera *Rhizobium*, *Bacillus*, and *Pseudomonas* and fungi of the genera *Penicillium* and *Galactomyces*. Microbes present in the rhizosphere manifest an alternative biotechnological solution to meet out P demands of plants sufficing sustainable agriculture production (Zaidi et al. 2009).

10.2.2.3 Potassium

Potassium (K) deficiency in plants can expedite both entry and spread of pathogens as well as insects (Amtmann et al. 2008). Microarray experiments have revealed strong repression of N transporters in K-deficient plants, which is quickly neutralized for an adequate supply of K to the plants. Under K-deficient conditions, the

pathogens and insects attack crop yield insignificantly affected by synergistic and antagonistic effects which trigger in plant under stress condition (Armengaud et al. 2004), while in some host plants symbiotic organisms may contribute to potassium buildup (Basak and Biswas 2012). Potassium-solubilizing microorganisms (KSM) in K plant nutrition play an important role (Meena et al. 2014). A large number of saprophytic bacteria (including *Paenibacillus* spp., *Bacillus edaphicus*, *Bacillus circulans*, *Acidithiobacillus ferrooxidans*, and *Bacillus mucilaginosus*) and fungal strains (*Aspergillus terreus* and *Aspergillus* spp.) conciliate K solubilization in soil system.

The mechanisms associated with KSM are complexolysis, chelation, exchange reactions, acidolysis, and organic acid production. Plant can easily take up K for growth and development after its solubilization by KSM.

10.3 Plant-Microbe Interactions in Soil

Plants are dependent on soil for their survival; however, plants along with their microbial associates also play a significant role in either modification or formation of soil (Pate et al. 2001). Microorganisms play an important role in biogeochemical nutrient cycling and being associated with plant roots also deliver nutrients and suppress pathogens, which sustains not only plant and animal health but life on earth as well. *Bacillus subtilis* is known to produce more than 20 antibiotics and has potential toward disease suppression. Its efficacy is well reported in various crop plants like chili, tomato, brinjal, etc., to control multiple pathogens like *C. capsici*, *Pythium aphanidermatum*, *C. gloeosporioides*, *Colletotrichum acutatum*, and *R. solani* (Abdul et al. 2007). *Streptomyces* species can also be used as inoculants, due to plant growth-promoting abilities. It produces several lytic enzymes which brings breakdown of insoluble organic polymers, thereby generating nutrients easily available to plants (Vurukonda et al. 2018).

10.3.1 Implication of the Soil Microbiome on Sustainable Agriculture and Food Security

There is an increasing demand of food to feed the present population which is increasing by leaps and bounds, and for this the world is looking toward a sustainable agriculture era. Global green revolution targets to the process of increase in food production, imparting food security and improving quality of food so as to sustain growth in population without compromising with environmental safety (Gupta 2012).

For development of sustainable agriculture, there is a constant need for identification, isolation, and utilization of microbes as a potential substitute for chemical inputs for crop protection (Bhattacharyya and Jha 2012). An increase in richness of soil microbial species has been shown to predict plant health and productivity (Van der Heijden et al. 2008). The potential microbial isolates may be formulated

Table 10.1 Commercial products of plant growth-promoting rhizobacteria in plant health and disease management

Bioagent	Trade name/formulation
Pseudomonas syringae ESC-100	BIO-save 10,11,100,110,1000 and 10 LP
Pseudomonas chlororaphis	Cedomon
Pseudomonas cepacia	Intercept
Streptomyces griseoviridis K61	Mycostop
B. subtilis + *B. amyloliquefaciens*	Bio-YIELD
Pseudomonas spp. + *Azospirillum* spp.	Biojet
Bacillus pumilus GB 34	Concentrate; YieldShield
B. pumilus QST2808	Sonata ASO, Ballard
B. subtilis GB03	Companion, System 3, Kodiak, Kodiak HB, Epic
Bacillus amyloliquefaciens GB99	Quantum 4000
Bacillus licheniformis SB3086	EcoGuard, Green Releaf
Burkholderia cepacia	Blue Circle, Deny, Intercept
P. fluorescens A506	BlightBan A506, Conquer, Victus
Agrobacterium radiobacter strain K1026	Nogall
A. radiobacter strain K84	Galltrol, Diegall
Azospirillum brasilense/Azotobacter chroococcum	Gmax Nitromax
A. brasilense	Azo Green
B. subtilis MB1600	BaciGold, Histick N/T, Subtilex
B. subtilis strain FZB24	Rhizo-Plus, Serenade, Rhapsody, Taegro, Tae-Technical
Bacillus chlororaphis 63–28	AtEze
Bacillus cereus BPO1	Pix Plus

employing different inorganic and organic carriers either through submerged or solid-state fermentation technologies (Table 10.1).

10.3.2 Interactions Between Plant and Microbes: A Common Signaling Pathway for Infection by Rhizobia and Arbuscular Mycorrhizal Fungi

Symbiotic bacteria associated with leguminous plants are well known as rhizobia. For sustainable agriculture a study of the mechanism and physiology of nodulation is required because biological fixation of nitrogen and its availability to plants is a complex and dynamic process. Plants have evolved a common signaling system for symbiosis which promotes uptake of nutrient despite the difference between symbionts. Arbuscular mycorrhizal fungi (AMF) infection leads to the initiation of nodulation process in *L. japonica* involving seven genes (Kistner et al. 2005). Arbuscular mycorrhizal fungi are important components of the soil microbes, and they interact with the other microorganisms present in the rhizosphere (Bowen and Rovira 1999). AM may attribute changes in certain nutritional and/or plant physiology and physical properties of the soil rhizosphere. This may lead to modification in colonization

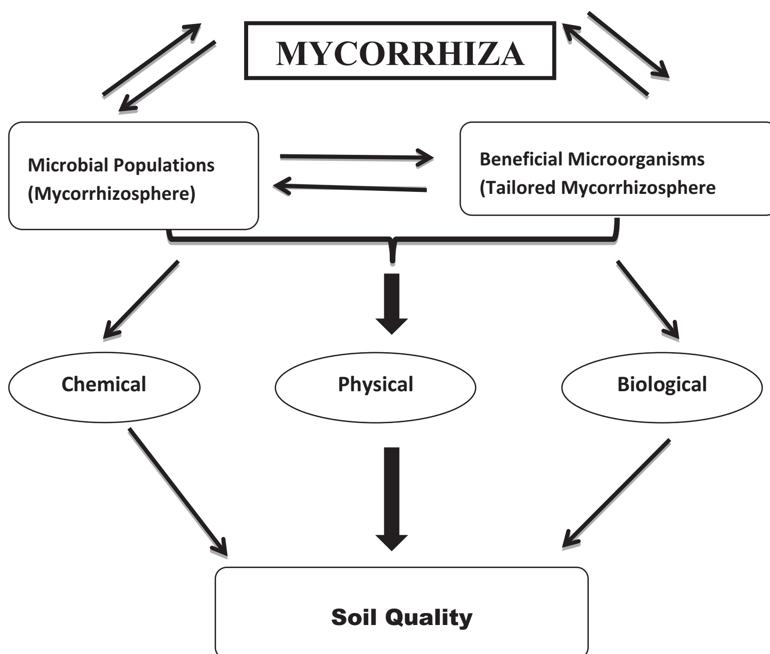

Fig. 10.1 Arbuscular mycorrhizal fungi interact with natural and genetically engineered microorganisms in the mycorrhizosphere, thus affecting soil properties and quality

patterns of this region by soil microbiota following the process popularly known as mycorrhizosphere effect (Gryndler 2000). Interaction of AMF with natural and introduced microorganisms in the mycorrhizosphere may lead to progressive changes in soil properties and quality (Fig. 10.1). Soil microorganisms may affect formation of AM and its functioning markedly (Barea et al. 2002).

10.3.3 Role of Soil Microbes in Soil Health and Plant Productivity

Soil ecosystem is very complex which hosts fungi, animals, Protista, and bacteria but is also viewed as nutrient source to plants (Bonkowski et al. 2009; Muller et al. 2016). Fair microbial diversity is a good indicator of soil health (Nielsen and Winding 2002). Inoculation of potentially beneficial microorganisms in addition to those already present in soil may optimize nutrient uptake by plants (Kirankumar et al. 2008), attribute to abiotic stress resistance (Selvakumar et al. 2012), improve plant growth (Cummings 2009; Guinazu et al. 2009), and decrease disease incidence (De Vleesschauwer and Hofte 2009).

Living microorganisms are not only self-sustaining and dynamic but also can encounter the problem of pathogens and pests evolving resistance to the treatments, thereby decreasing the need for repeat applications (Lucas 2011). The

advancements in this area over the last several years have shown the potential for PGPR applications in improvement of agricultural production and sustainability. Plants are known to interact with a diverse variety of PGPRs that confer drought and salt tolerance (Dimkpa et al. 2009; Zhang et al. 2010), thereby improving plant's own nutrient acquisition mechanisms, increasing disease suppression (Chithrashree et al. 2011), and increasing photosynthetic efficiency (Xie et al. 2009) and overall plant growth. PGPR traits are known to produce biofuel crops in areas scored unsuitable for agricultural production (Tilman et al. 2009), a situation in which drought and salt tolerance could become especially important.

10.4 Metagenomics

Our physical environment is a well-known reservoir of microbial genetic diversity (Robe et al. 2003). Soil microorganisms, although well adapted to their environment, cannot be cultured under normal laboratory conditions. Moreover, more than 98% of microorganisms in the environment are unculturable, and majority of microbiologists focus specifically on culturable microbes for the discovery of novel biomolecule(s). In the last two decades, the field of metagenomics has revealed new approach toward accessing the biosynthetic machinery of uncultured bacteria, thereby encompassing traditional molecular methods which rely on cultivation. Metagenomic approach, whereby the whole DNA is directly extracted from the environmental samples and sequenced employing next-generation sequencing platforms (Illumina, SOLiD, PacBio, Oxford NanoPore), may be used to circumvent the challenges associated with the isolation of unculturable microorganisms (Maphosa et al. 2012).

Metagenomics initially involves the isolation of total or enriched DNA directly from the environment (eDNA) and its cloning into a easily cultivated host (Handelsman 2004; Miao and Davies 2009). Recent development in next-generation sequencing (NGS) technologies permit isolated eDNA to be sequenced and analyzed directly from environmental samples (Shokralla et al. 2012). This allows investigation of the biosynthetic potential of an uncultured microorganism which further involves the construction of an eDNA library in a suitable host as shown in Fig. 10.2.

10.4.1 Bioprospection of Novel Molecules

Metagenomic analysis is gradually emerging as a promising tool to derive different biomolecules from an uncultivable diversity of our environment. Phylogenetic studies have revealed that with the help of molecular tools, the representation of cultured microbes is very small (<1%) of the real microbial diversity, because it is very difficult to mimic the natural habitat conditions under synthetic culturing conditions (Amann et al. 1995; Zengler et al. 2002) Gillespie et al. (2002). New technologies

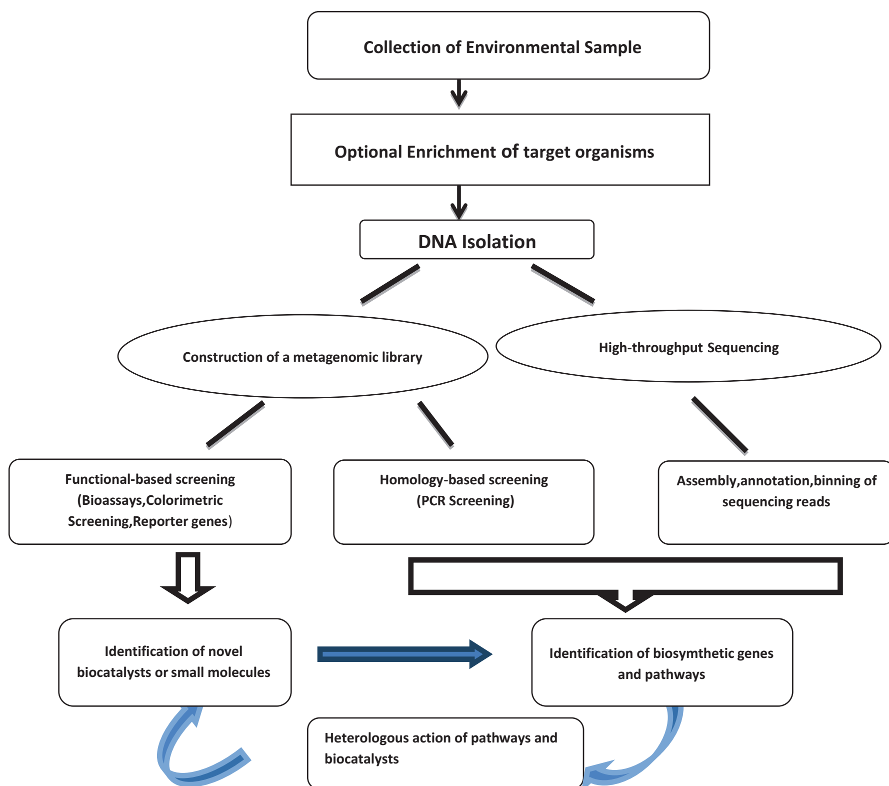

Fig. 10.2 Steps involved in a typical metagenomic workflow

like NMR- or MS-based metabolomics are efficient to find novel antibiotics microbial cultures (Wu et al. 2015). Using metagenomics approach has reported two important antibiotic compounds, namely, turbomycin A and B. Similarly, biocatalysts play vital roles in all biological organisms and are the essential class of proteins. Through different metagenomic and metabolomic tools, novel biocatalysts can be explored (Piel 2011; Lorenz et al. 2002). Metagenomics may be used as a tool for identification of various enzymes like amylases, lipases, proteases, xylanases, and cellulases (Nazir 2016). Various techniques find its application in detection of novel enzymes including in silico capillary electrophoresis electrospray ionization mass spectrometry (CE-MS), activity-based protein profiling, activity-based metabolomic profiling, and X-ray crystallography (Fig. 10.3).

10.4.2 Metagenomics: Ecological Inferences

There is a symbiotic relationship between eukaryotes and various groups of microorganisms, and they mutually compete for nutrients to produce energy (Handelsman

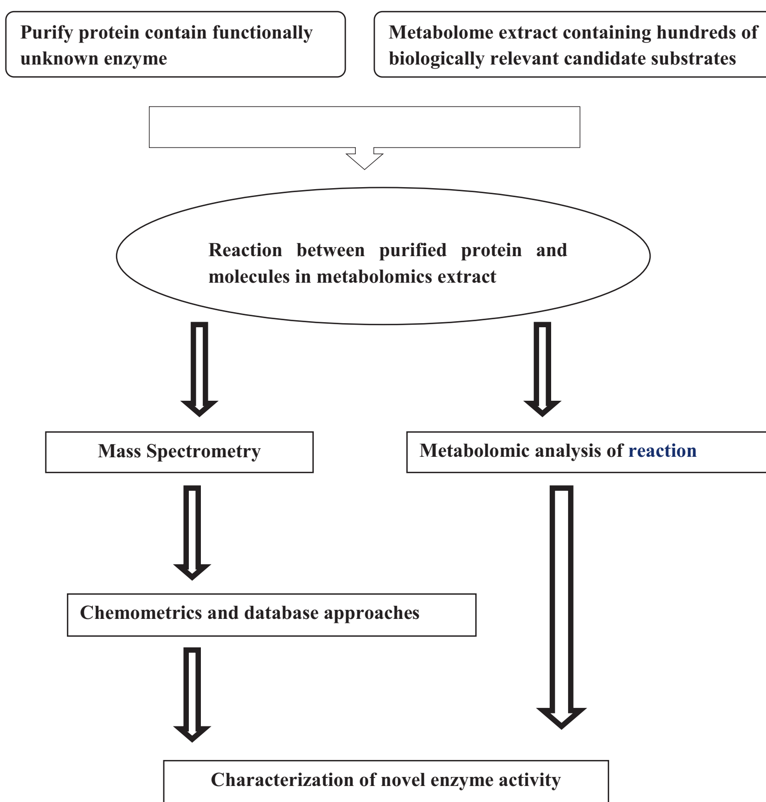

Fig. 10.3 Steps for novel enzymatic activities characterization using metabolomic approach

2004). Sponges are known to contain enormous number of bacteria within their tissues accounting for the 40–60% of the total biomass (Hentschel et al. 2006; Preston et al. 1996). They act as filter-feeding organisms and produce secondary metabolites by processing huge volume of seawater per day and have eventually evolved as biofouling and chemical defense against predator.

Metagenomics is emerging as a potential tool for exploring microbial diversity besides forming the basis of genomic studies so as to link functional and phylogenetic relationship between microbial diversity and environment. It is useful in determining 16 s rRNA gene sequences, and different microorganisms have been identified from diverse environment such as extreme desert environment, sponges, acid mine drainage, etc. (Singh et al. 2009). *E. coli* is universally used as a host strain during screening of soil-derived metagenomic DNA for confrontation as novel biocatalysts and small molecules (Majerník et al. 2001; Knietsch et al. 2003). It is used commercially in separation, downstream processing, batch production, and industrial fermentations.

10.4.3 Metagenomics: Environmental Bioremediation

A strain of *Pseudomonas stutzeri*, isolated using metagenomic approach from Indian coal bed, could solubilize coal and is found to produce a large amount of biosurfactant when coal was added to a medium (Rogers and McClure 2003). In another study on metaproteogenomic, the effect of long-term metal exposure on the bacterial communities was studied (Gillan et al. 2014). The study was conducted in two freshwater sites, and the samples were taken at different magnitudes in metal levels, following comparison between the two site samples using shotgun metaproteogenomics which resulted in a total of 69–118 Mbp of DNA and 943–1241 proteins (Devarapalli and Kumavath 2015). Upon observation the two communities were found to be functionally similar. Through application of advanced metagenomic approaches, significant genetic differences were observed for three categories: virulence, defense mechanisms and synthesis of exopolymeric substances, and elements involved in horizontal gene transfer. In addition to sequencing-based approaches, several microarray-based techniques have also been developed (Hazen et al. 2013). PhyloChip and GeoChip are the two most commonly used microarray technologies. PhyloChip is a 16S rRNA-based microarray able to probe the diversity of 10,993 subfamilies in 147 phyla (Hazen et al. 2010). GeoChip is a functional gene microarray able to probe the diversity of 152,414 genes from 410 gene categories (Zhou et al. 2013).

10.5 Conclusion

Coexistence of plants and microorganisms dates back to several million years. Plants with their rhizospheric populations maintain complex interaction, which is crucial for assimilation of nutrient and development and activation of defense mechanisms. Such associations are mutually beneficial because plants and microorganisms can communicate among themselves through various signaling mechanisms. Transkingdom signaling between plants and bacteria has been found to be based on small lipid signals (i.e., *N*-acyl-L-homoserine lactones (AHLs)) and is revealing its diverse roles in healthy plants. The area of plant-microbe interactions is undoubtedly likely to provide excellent examples of the molecular mechanisms involved in the interaction. A further exploration of such interactions by global gene expression analyses along with proteomic strategies and the identification of plant mutants defective on signal perception/transduction will definitely help to increase our knowledge on the mechanisms on plants and beneficial microbial cross talks.

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Role of Microbial Genomics in Crop Improvement

11

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Abstract

Genomic sequencing and analysis are in a period of exponential growth. The nearly complete human genome sequence is the cornerstone of genome-based biology and provides the richest intellectual resource in the history of biology. The availability of entire genome sequences marks a new age in biology because it has the potential to open innovative and efficient research avenues. Determination of entire genome sequences is only the first step in understanding the inner workings of an organism. The next critical step is to elucidate the functions of these sequences and give biochemical, physiological, and ecological meaning to the information. Sequence analysis indicates that the biological functions of substantial portions of complete genomes are unknown. Defining the role of each gene in the complex cellular machine and network is a formidable task. In addition, genomes contain hundreds to thousands of genes many of which encode multiple proteins that interact and function together as multicomponent systems or apparatuses for accomplishing specific cellular processes. The products of many genes are often coregulated in complex signal transduction networks and understanding how the genome functions as a whole to give life to complete organisms presents an even greater challenge. In addition, gene functions, protein machinery, and regulatory networks cannot be identified solely by using traditional single-gene, single-protein approaches. Thus, many laboratories are addressing important questions in functional genomics research by integrating genomic, proteomic, genetic, biochemical, and bioinformatic approaches.

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Consequently, areas in functional genomics and associated genomic technology are developing very rapidly. Rapid exchange of knowledge and the establishment of critical collaborations are vital to remaining on the cutting edge of this field. All of these meetings have attracted leading scientists and institutions involved in genome sequencing, microbial functional genomics, and genomic and proteomic technologies.

Keywords
Microbes · Genomics · Crop improvement

11.1 Introduction

The agricultural production is expected to increase by at least 70% by 2050. At the same time, people are becoming aware that sustainable agricultural practices are fundamental to meet the future world's agricultural demands (Altieri 2004). This is why modern agriculture is being implemented on a global scale and diverse research approaches are being undertaken addressed to meet environmental and economical sustainability issues, trying to save at most as possible usage of nonrenewable natural resources. A recommended approach is that based on exploiting the role of microbial communities for a sustainable and healthy crop production while preserving the biosphere. Actually, soil microorganisms play fundamental roles in agriculture mainly by improving plant nutrition and health, as well as soil quality (Lugtenberg 2015). The microbial genomics is an interdisciplinary field of science focusing on the structure, function, evolution, mapping, and editing of genomes. A genome is an organism's complete set of DNA, which includes all of its genes. In contrast to genetics which refers to the study of individual genes as well as their roles in inheritance, genomics aims at the collective characterization and quantification of genes, which direct the production of proteins with the assistance of enzymes and messenger molecules. In turn, proteins make up body structures such as organs and tissues as well as control chemical reactions and carry signals between cells. Genomics also involves the sequencing and analysis of genomes through uses of high-throughput DNA sequencing and bioinformatics to assemble and analyze the function and structure of entire genomes. The analysis of an organism's complete DNA sequence has been one of the most transformative influences on biological studies. The knowledge of complete genome sequences of organisms are basically important for understanding the functions of individual genes and their networks, for defining evolutionary relationships and processes, and for revealing previously unknown regulatory mechanisms that coordinate the activities of genes. These genomics-based approaches are having a profound influence on both human disease diagnostics and treatment and, equally importantly, on the improvement of crops for food and fuel production.

11.2 What Is Microbial Genome?

Microbial genomes are widely variable and reflect the enormous diversity of bacteria, archaea, and lower eukaryotes. Bacterial genomes usually consist of a single circular chromosome, but species with more than one chromosome (e.g., *Deinococcus radiodurans*), linear chromosomes (e.g., some *Bacillus subtilis* strains), and combinations of linear as well as circular chromosomes (e.g., *Agrobacterium tumefaciens*) also exist. Plant-associated microbes play critical roles in agricultural and food safety and security and in the maintenance of ecosystem balance. Some of these diverse microbes, which include viruses, bacteria, oomycetes, fungi, and nematodes, cause plant diseases, while others prevent diseases or enhance plant growth. Despite their importance, less than 6% of the microbes whose genomic sequence has been completed and made publicly available were plant-associated microbes. The study of microbial genomes helps us to better understand the broader biology of bacteria and how their genetic composition contributes to their tangible characteristics. The study of genomics is also important to infer the evolution of bacteria. Bacteria often evolve not just through small, single nucleotide level changes but through quantum evolutionary events. These include through the transfer of plasmids between species and also the transposition of large genetic elements within single cells. Understanding these processes allows us to determine the origins of bacteria and map the transfer of genes such as those conferring antibiotic resistance.

11.3 The Importance of Microbiology in Crop Yield

The knowledge of how microbes interact with plants is essential to the development of effective, environmentally sound, chemically based strategies for disease control. The heavy use of antibiotics targeted to human or animal pathogens and changes in pathogen biology more and more frequently render some of these chemicals ineffective. Furthermore, increased regulatory policies are restricting the use of existing agrochemicals for pathogen control. With genome information, multiple tactics for control of pathogens could be developed. For example, the identification of more precise targets in the pathogen may allow for design of more specific and effective chemicals that are environmentally benign. The powerful automated sequence technologies currently available and the advances in bioinformatics have made the task of sequencing entire genomes of organisms almost routine. The sequenced genomes of a few model organisms are already enabling a wide variety of new discoveries, including new genes and metabolic pathways and insights into the mechanisms of microbial pathogenesis. The continuous improvement of bioinformatics tools is enhancing the discovery power of these sequences. With these exciting advances, the means are now available to generate sequence and function information, as are the tools to use that information to understand the basic biology of microorganisms that cause or prevent diseases on plants.

By enhancing our knowledge base, genome analyses will provide tools to abrogate the problems caused by plant pathogenic microbes through genetically based approaches and will allow development of improved beneficial microbes. Historically, we have studied the molecular basis for interactions between plants and microbes using a gene-by-gene approach and have used host plant resistance as a major control approach. Now, structural and functional genomic analyses will increase the speed of identification of genes involved in host-pathogen interactions and will allow genome-wide approaches to understanding the role of a gene or pathway in interactions with plants. Some genes will potentially be useful as sources of pathogen-derived resistance, as has already been demonstrated for many viral diseases. Other genes may be involved in activating plant defense responses. Comparisons of genomes of related strains or species will provide an understanding of the evolution of microbial genomes, particularly as they evolve in associations with plants. Researchers exploiting comparative genomics will be able to predict the molecular basis of how some microbes have evolved to form intimate biotrophic associations with plant cells, whereas others inhabit intercellular spaces, and still others colonize only the vascular systems of their hosts. Comparisons of sequences within and between species will also provide information to develop accurate diagnostic tools.

Plants, bacteria, and fungi have coexisted in soil in a symbiosis for millions of years, and they both benefit greatly from symbiosis. Healthy microbial communities perform a variety of vital ecosystem functions, such as pathogen suppression and regulation of nutrient availability. For example, microorganisms make essential elements such as nitrogen and phosphorus available in exchange for carbon provided by plants. In plant-microbe interactions, two symbiotic systems have been extensively studied and well understood: arbuscular mycorrhizal (AM) symbiosis and root nodule (RN) symbiosis. Microorganisms also do many other things like decomposing organic matter such as crop residues and releasing the nutrients, plant growth control, as well as maintaining soil structure and good hydrology.

11.4 Microbial Genomics Methods to Promote Crop Production

The productivity of plants largely depends on the soil quality in which microorganisms play a major role. One way to develop improved sustainable crop yield is to enhance the beneficial plant-associated microbiome. And microbial genomics represents a series of powerful tools to differentiate beneficial, neutral, and harmful microorganisms. The common microbial genomics technologies include 16S/18S/ITS amplicon sequencing, metagenomics, metatranscriptomics, microbial whole-genome sequencing, complete plasmid sequencing, and microbial single-cell sequencing. The first three methods are genetic investigations into microbial communities, while the last three methods represent genetic studies of individual microorganisms.

Microbial genomics approaches can promote crop yield through soil microbial diversity analysis, functional microbial genomics, biological products development, and bacterial genome modification by utilizing high-throughput and high-resolution genomics technology platforms. Both next-generation sequencing and PacBio SMRT sequencing systems are excellent platforms for microbial research. In addition to generating long-length reads, PacBio SMRT systems are able to characterize methylation with PacBio analytical tools.

11.5 Microbial Diversity Analysis

The abundance and diversity of soil microbial communities are indicators of crop health. A healthy soil microbial community supports a balanced and sustainable ecosystem. The 16S/18S/ITS sequencing is a powerful and common method. It can explore soil microbial abundance and diversity quickly and efficiently. After generating various isoforms of targeted genes, you are able to determine taxonomic composition of soil microbial communities and understand the probable cause of low crop yield by comparing microbiomes between high-yield and low-yield crops. Artificial manipulation of soil microbial community composition may increase crop yield, such as addition of beneficial microbial strains or removal of pathogenic microbes.

11.6 Functional Microbial Genomics

Understanding the taxonomic composition of soil microbial communities is far from enough. Functional microbial genomics is crucial to elucidate microbiome functions; differentiate beneficial, neutral, and pathogenic microbes; as well as provide insights into improvement of soil quality and bacterial strains and biological products development. Functional microbial genomics allows researchers and farmers to select important microbial strains that play important roles in achieving the maximum yield potential of a crop.

11.7 Microbial Products Development

Metagenomics, metatranscriptomics, microbial whole-genome sequencing, and single-cell sequencing help scientists to develop microbial products such as probiotics. Probiotics represent living bacteria or yeast that are good for environment. Microbial products have been used for sustainable agriculture contributing to increase crop yield by optimizing beneficial bacteria or protecting plants from pests and disease. Compared with inorganic/organic fertilizers and pesticides, microbial products are more effective and environmental-friendly.

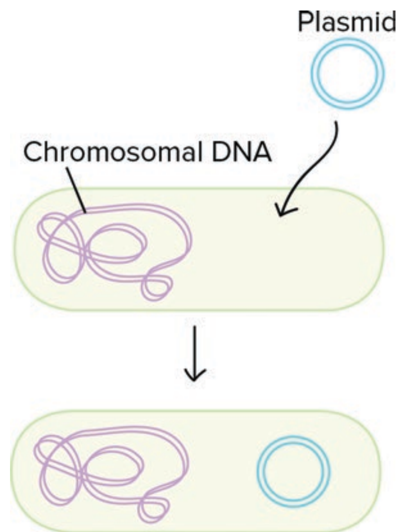
11.8 Microbial Genome Modification

After characterizing microbiome functions and genomes by high-throughput sequencing and phenotype analysis, the appropriate microbial strains with activities of interest can be selected, and their genomes can be altered in a very targeted manner, with the power of genetic engineering. Compared with traditional random methods, it is more efficient and sometimes necessary to use targeted techniques for strains modification to improve the relevant characteristics of the strain.

11.9 Basic Procedure of Microbial Genome Transfer

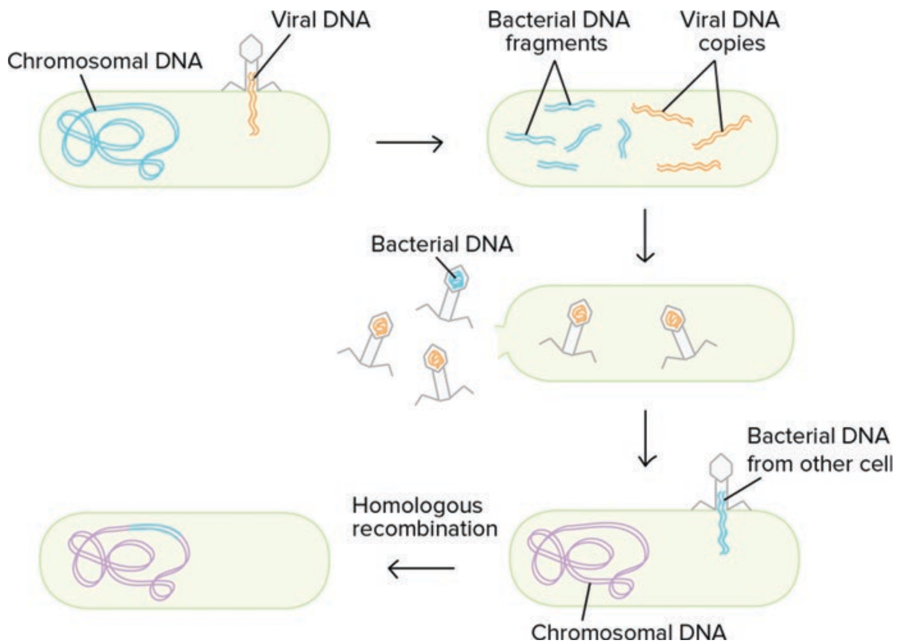
11.9.1 Transformation

In transformation, a bacterium takes in DNA from its environment, often DNA that's been shed by other bacteria. In a laboratory, the DNA may be introduced by scientists. If the DNA is in the form of a circular DNA called a plasmid, it can be copied in the receiving cell and passed on to its descendants.



11.9.2 Transduction

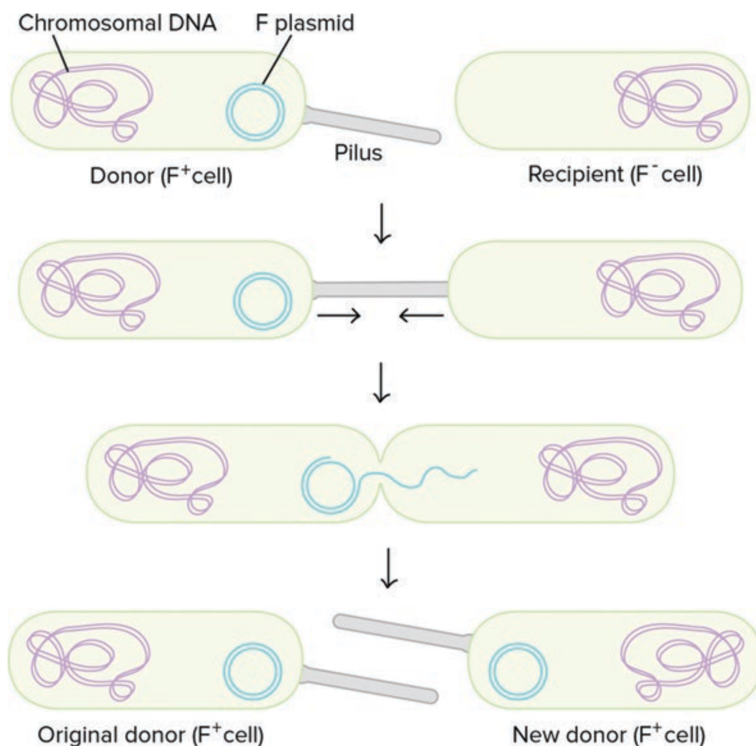
In transduction, viruses that infect bacteria move short pieces of chromosomal DNA from one bacterium to another "by accident." The viruses that infect bacteria are called bacteriophages. Bacteriophages, like other viruses, are the pirates of the biological world – they commandeer a cell's resources and use them to make more bacteriophages.



Archaea, the other group of prokaryotes besides bacteria, are not infected by bacteriophages but have their own viruses that move genetic material from one individual to another.

11.9.3 Conjugation

In conjugation, DNA is transferred from one bacterium to another. After the donor cell pulls itself close to the recipient using a structure called a pilus, DNA is transferred between cells. In most cases, this DNA is in the form of a plasmid.



Donor cells typically act as donors because they have a chunk of DNA called the fertility factor (or F factor). If the F factor is transferred during conjugation, the receiving cell turns into an F^{++} donor that can make its own pilus and transfer DNA to other cells.

11.10 Transposable Elements

Transposable elements are also important in bacterial genetics. These chunks of DNA “jump” from one place to another within a genome, cutting and pasting themselves or inserting copies of themselves in new spots. Transposable elements are found in many organisms. In bacteria, transposable elements sometimes carry antibiotic resistance and pathogenicity genes (genes that make bacteria disease-causing). If one of these transposable elements “jumps” from the chromosome into a plasmid, the genes it carries can be easily passed to other bacteria by transformation or conjugation. That means the genes can spread quickly through the population.

Benefits of Gene Transfer Technologies

1. Provide resistance against viruses.
2. Acquire insecticidal resistance.

3. Strengthen the plant to grow against bacterial diseases.
4. Develop the plants to grow in draught.
5. Engineer plants for nutritional quality.
6. Make the plants to grow in various seasons.
7. Herbicide-resistant plant can be made.
8. Resistance against fungal pathogens.
9. Engineer plants for abiotic stress tolerance.
10. Delayed ripening can be done.

11.11 Application of Microbial Gene Transfer

The most widely used technique for plant transformation is based on *Agrobacterium*, in which novel genes, linked to the Ti or Ri plasmid T-DNAs, are inserted into the host plant cells during T-DNA transfer (Hooykaas and Schilperoort 1985). This approach has been used to transform numerous plants. Transformation of rice is the staple food for more than one third of world's population. To feed the growing world population, it is the requirement to increase the total food production. Although the world food supply has more than doubled since the onset of the green revolution, still there is a need to improve the quantity as well as quality. Ballistic was successfully used for transformation of immature embryos of rice. Reports were also made regarding the transformation of indica and javanica rice in addition to japonica rice (Christou et al. 1991). Patnail and Khurana (2001) were the first to engineer japonica rice through electroporation with modified d-endotoxin gene (cry) from *Bacillus thuringiensis*. It was found that the R2 generation of transgenic rice was more resistant to insects than wild-type plants. Later, Wunn et al. (1996) obtained transgenic indica rice cultivar IR58 expressing a synthetic cryIA (b) gene driven by 35S promoter through particle bombardment. Transgenic rice plants harboring the cloned gene displayed high levels of resistance. The gene has been found to be effective against several isolates. A coat protein-mediated resistance to viruses, introduced in rice via protoplast transformation (Hayakawa 1992), was transferred to maize and barley via particle gun bombardment.

11.12 Microbe-Mediated Mitigation of Abiotic Stress

Microbial interactions with crop plants are key to the adaptation and survival of both the partners in any abiotic environment. Induced systemic tolerance (IST) is the term being used for microbe-mediated induction of abiotic stress responses. The role of microorganisms to alleviate abiotic stresses in plants has been the area of great concern in the past few decades (Souza et al. 2015). Microbes with their potential intrinsic metabolic and genetic capabilities contribute to alleviate abiotic stresses in the plants (Gopalakrishnan et al. 2015). The role of several rhizospheric occupants belonging to the genera *Pseudomonas*, *Azotobacter*, *Azospirillum*,

Rhizobium, *Pantoea*, *Bacillus*, *Enterobacter*, *Bradyrhizobium*, *Methylobacterium*, *Burkholderia*, and *Trichoderma* and cyanobacteria in plant growth promotion and mitigation of multiple kinds of abiotic stresses has been documented. Recently have demonstrated the role of *Trichoderma harzianum* on stress mitigation in rice genotypes due to upregulation of aquaporin, dehydrin, and malondialdehyde genes along with various other physiological parameters. Rhizobacteria-induced drought endurance and resilience (RIDER) that includes changes in the levels of phytohormones, defense-related proteins and enzymes, antioxidants, and epoxy polysaccharide has been observed for microbe-mediated plant responses. Such strategies make plants tougher toward abiotic stresses (Kaushal and Wani 2016). The selection, screening, and application of stress-tolerant microorganisms, therefore, could be viable options to help overcome productivity limitations of crop plants in stress-prone areas. Enhanced oil content in NaCl affected Indian mustard (*Brassica juncea*) was reported by *Trichoderma harzianum* application which improved the uptake of essential nutrients, enhanced the accumulation of antioxidants and osmolyte, and decreased Na^+ uptake (Ahmad et al. 2015). Parallel to such reports, upregulation of monodehydroascorbate reductase in *Trichoderma*-treated plants was demonstrated. It was also confirmed by mutant studies that *Trichoderma* ameliorates salinity stress by producing ACC-deaminase. Studies using the prokaryotic organism *Escherichia coli* suggest that the synthesis of organic solutes may be the crucial step for salt tolerance because the first described bacterial halotolerance gene (proB-74) determines the over accumulation of proline. In the eukaryotic microorganism *Saccharomyces cerevisiae*, however, potassium homeostasis seems to be the most critical response to salt stress. The first halotolerance gene isolated from this organism (HAL1) seems to modulate potassium transport, increasing the intracellular level of this cation in NaCl-containing media. The existence of plant homologues to HAL1 indicates that yeast may be a useful model for the genetics of salt tolerance in plants (Serrani and Gaxiola 1994) (Table 11.1).

11.13 Progress in Sequencing of Crop Genome Using BAC

Advances in sequencing crop genomes have mirrored the development of sequencing technologies (Table 11.2). Until 2010, to access crop genomes such as rice, poplar, and maize, Sanger sequencing of bacterial artificial chromosome (BAC)-based physical maps was the predominant approach used (Schnable et al. 2009). The rice genome comprises complete sequences of individual BACs assembled into physical maps that are anchored to genetic maps, whereas for maize, the sequences of individual BACs were not completely finished. For sorghum and soybean (Paterson et al. 2009; Schmutz et al. 2010), whole-genome shotgun (WGS) reads of libraries of randomly sheared fragments of different sizes and of BAC end sequences (BES) were assembled with powerful assembly algorithms such as ARACHNE (Hood et al. 2004; Batzoglou 2002). Physical maps of BACs provide a good template for completing gaps and errors, but genome coverage of physical maps can be nonrepresentative due to cloning bias. In addition, intensive handcrafting is required to assess physical map integrity and to close gaps; this effort scales directly with

Table 11.1 Microbe-mediated abiotic stress tolerance in plants

Abiotic stress	Microbe inoculation	Plant	Tolerance strategy	Reference
Salt	*Bacillus subtilis* GB03	*Arabidopsis thaliana*	Tissue-specific regulation of sodium transporter HKT1	Zhang et al. (2008)
Salt	*Pseudomonas simiae*	*Glycine max*	4-Nitroguaiacol and quinoline promote soybean seed germination	Vaishnav et al. (2016)
Salt	Root-associated plant growth-promoting rhizobacteria (PGPR)	*Oryza sativa*	Expression of salt stress-related RAB18 plant gene	Jha et al. (2014)
Salinity	*Glomus clarum*, *Glomus etunicatum*	*Vigna radiata*, *Capsicum annuum*, *Triticum aestivum*	Decreased Na^{+} in root and shoot and increased concentration of K^{+} in root	Rabie (2005), Daei et al. (2009), Kaya et al. (2009)
Drought	*Burkholderia phytofirmans*, *Enterobacter* sp. FD17	*Zea mays*	Increased photosynthesis, root and shoot biomass under drought conditions	Naveed et al. (2014)
Drought	*Bacillus thuringiensis* AZP2	*Triticum aestivum*	Production of volatile organic compounds	Timmusk et al. (2014)
Heat	*Bacillus amyloliquefaciens* *Azospirillum brasilense*	*Triticum aestivum*	Reduced regeneration of reactive oxygen species, preactivation of heat shock transcription factors, changes in metabolome	El-Daim et al. (2014)
Arsenic toxicity	*Staphylococcus arlettae*	*Brassica juncea*	Increased soil dehydrogenase phosphatase and available phosphorus	Srivastava et al. (2013)
Zn toxicity	*Pseudomonas aeruginosa*	*Triticum aestivum*	Improved biomass, N and P uptake and total soluble protein	Islam et al. (2014)
Zn toxicity	*Pseudomonas brassicacearum*, *Rhizobium leguminosarum*	*Brassica juncea*	Metal-chelating molecules	Adediran et al. (2016)

genome size and complexity. The sorghum genome (Paterson et al. 2009) was the first crop genome to be sequenced completely by the exclusive use of WGS sequence assemblies, which were then assessed for integrity using high-density genetic maps and physical maps. This pioneering analysis showed that scaffolds of Sanger sequence assemblies accurately span extensive repetitive DNA tracts and extend into telomeric and centromeric regions. The larger soybean genome was subsequently sequenced to similar high standards. The soybean genome is thought to be pseudo-diploid, derived from the diploidization of an allopolyploid in the past 50 million years (Schmutz et al. 2010; Gill et al. 2009), and this project successfully

Table 11.2 Progress in crop genome sequencing

Species (common name)	Genome size	Sequence strategy	Assembly features	Reference
Oryza sativa (rice)	389 Mb	BAC physical map, Sanger sequencing	Essentially complete chromosome arm coverage	Tuskan et al. (2006)
Sorghum bicolor (sorghum)	700 Mb	WGS, Sanger sequencing	229 scaffolds containing 97% of the genome, 88% of sequence genetically anchored	Schmutz et al. (2010)
Zea mays (maize)	2300 Mb	BAC physical map	2048 Mb in 125,325 contigs forming 61,161scaffolds	Jaillon et al. (2007)
Glycine max (soybean)	1115 Mb	WGS, Sanger sequencing	397 scaffolds containing 85% of the genome, 98% of sequence genetically anchored	Hood et al. (2004)
Brassica rapa (Chinese cabbage)	485 Mb	WGS, Illumina, BAC and Sanger sequencing	288 Mb in scaffolds, 90% of the assembly genetically anchored	Wang et al. (2011)
Cajanus cajan (pigeon pea)	833 Mb	WGS, Illumina	137,542 scaffolds containing 73% of the genome	Prochnik et al. (2012)
Gossypium raimondii (cotton)	880 Mb	WGS, Illumina	4715 scaffolds containing 85% of the genome, 73% of the assembly genetically anchored	Xu et al. (2012)
Hordeum vulgare (barley)	5100 Mb	WGS, Illumina, BAC physical map, BAC sequence	Physical map (4.98 Gb), BAC sequence (1.13 Gb), WGS assemblies (1.9 Gb); integrated by physical map and syntenic order	Paterson et al. (2012)
Triticum aestivum (bread wheat)	17,000 Mb	WGS	Orthologous group assembly, 437 Mb	Mayer et al. (2012)

demonstrated that WGS assemblies are not confounded by large-scale genome duplication events.

The *Brassica* genomes are among the most challenging to sequence with respect to achieving large-scale assemblies because they have undergone three recent whole-genome duplications followed by partial diploidization (Town et al. 2006). Table 11.2 shows progress in sequencing two much larger Triticeae genomes, those of diploid barley (5100 Mb) (Mayer et al. 2012) and hexaploid bread wheat (17,000 Mb) (Brenchley et al. 2012). Both the exceptional scale and high repeat content (approximately 80%) of these genomes provide significant challenges to straightforward WGS sequencing and assembly, with genes being separated by hundreds of kb of repeats such as nested retro-elements (Choulet et al. 2010).

11.14 Future Perspective

In recent years, advancements in high-throughput multiomics technologies and computational integration have helped us to understand plant-microbiome interactions across scales and decipher individual signal molecules, proteins, genes, and gene cascades to connect them with functional gene pathways. Technological advancements have facilitated the understanding of gene editing systems, RNAi-mediated gene silencing, mutant technology and proteomics, and metabolite profiling to reveal interactive networks that advanced our understanding of microbe-mediated strategies of plant growth promotion and biocontrol. Advances in automation and large-scale bioinformatics have increased the repertoire of available genomes of plant-associated microbes and together with information on their interactions with host are helping researchers to discover valuable new microbial genes for improved plant growth and productivity. These advances not only provide a resource and conceptual framework for studying plant-microbiome interactions but also highlight many new potential plant-beneficial genomic circuits that could be targeted to improve plant productivity around the globe. Gene discoveries have resulted in the development of genetically engineered plants using novel microbial genes for disease resistance, herbicide tolerance, stress tolerance, and plant yield improvement. However, most of these breakthroughs were achieved by inserting a few genes or a combination of a few targets. Future research should focus on combining different strategies such as the multigenic approach to simultaneously incorporate more than one gene in transgenic plants. New tools and resources that can be applied to introduce complex heterologous pathways into plants hold the key to build synthetic genome clusters from microbiomes to enable the stacking and shuffling of disease resistance and stress tolerance traits between crop plants. New capabilities developed in trait discovery will further intensify the rate of novel gene discovery. For example, the CRISPR-Cas9-based forward genetic screen will help future studies of plant-microbiome interactions to transcend individual genes and become more holistic in approaches to elucidate plant-microbiome interactions and discover novel genes for biotechnological applications.

A wealth of genome information dramatically expands our understanding of a variety of microbial metabolic pathways available for novel traits. This leads to attempts to design and engineer microbial cell factories devoted to elucidate and investigate new metabolic pathways as well as the high-level production of the respective compounds allowing their characterization and application. Potential applications exist in the field of sustainable plant cultivation as several metabolites are known to improve plant health and growth. This can be effected via different mechanisms. Certain metabolites can directly trigger enhanced plant growth as signal molecules others can indirectly support plant growth by inhibition of plant pathogens or by shaping a beneficial microbiome around the plant.

The integration of microbial biofertilizers, biocontrol microbes, optimized microbiomes, soil amendments, and matching microbe-optimized crops for different soil types would be the ultimate goal for enhancing plant-microbe interactions. Clearly, this is a largely untapped area that deserves major research efforts, as it

holds the promise to improve crop yields and address food security in an environmentally friendly and sustainable manner. Overall, existing microbial technologies along with emerging microbiome and associated approaches offer new and more sustainable practices to increase agriculture productivity. Initial assessments highlight growing demand for microbial-based solutions for food security both from growers and consumers of the produce. However, significant scientific and technological challenges exist. If these challenges can be prioritized along with the improvement of regulatory framework, emerging microbial-based solutions can potentially transform sustainable agriculture. Given that agriculture has been central to the success of *Homo sapiens*, it is not surprising that such an approach can address multiple SDGs if implemented systematically.

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12 Current Status and Future Prospects of Omics Tools in Climate Change Research

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Abstract

Omics referring to a group of biological tools has greatly influenced today's world of modern research. Genomics, transcriptomics, proteomics, and metabolomics together they help to bring out the best of characters in plants and other organisms for its improvement and enhancement of important bioactive compounds. Genomic study for finding chromosome location, phenotypic analysis by QTL mapping, genome-wide association studies (GWAS), etc. are being practiced along with the development of genome editing by CRISPERCas9 for a variety of crop plants under stress conditions from the past few years. Studies made on yeast and *Arabidopsis*, transcriptome profiling, and microarray-based studies could detect the significant alteration of gene expression and some rare novel transcript to map out the physiological pathways. Mass spectroscopy-based approaches like NMR, MALDI, and GC-MS came into being to simplify protein and metabolite studies, its structure, and its function which reciprocate in many important biological signalling cascades. Physiological and morphological changes in an organism due to environmental stress are an ongoing issue and

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newest addition to the research field, and with time, changes in the entire genome are a matter to look into where only molecular approaches can answer it. Thus, in this chapter we tried to summarize various aspects of omics tools and its future scope which can be utilize in climate change research.

Keywords
Metagenomics · Metabolomics · Transcriptomics · Protein analysis · Sustainable agriculture · Human health

12.1 Introduction

Biological sciences have greatly advanced in solving unanswerable questions of various aspects over the years. Any changes in the biological functioning, science has its answer. There are factors which mainly influence such changes. Change in the gene leading to mutation is not only a chance factor but also mandated by external factors. In trend, climatic change, a subject of high interest of today's world, is one of the important facets and prime reason for the changing notion of biological constituent. Evolution could be a potential solution which could rescue populations from the effects of climatic change, but there is no hardcore evidence that this actually occurs (Franks and Hoffmann 2012). To know the exact reason of changes at gene level and ultimately at metabolic pathway "Omics" technologies will provide solution for these changes. Omics primarily deals with universal detection of genes (genomics), mRNA (transcriptomics), proteins (proteomics), and metabolites (metabolomics) (Horgan and Kenny 2011). Climatic change has always been a disadvantage to living organism leading to abiotic stress condition taking a catastrophic shape for human welfare. High or low temperature, high salinity, submergence conditions, etc. are some of the environmental factors which have greatly influenced researcher to carry out studies on various organisms but predominantly on plants. Studies in plant stresses have been carried out over the past decade, and the omics data is used to improve and elevate productivity of agricultural crop (Muthuramalingam et al. 2017). On the other hand, microbes, which are the most dominant inhabitant of environment, can survive in extreme condition and exhibit tremendous metabolic capabilities to mitigate abiotic stresses. It has always been known that microbes play an integral role in interaction with plant. Plant-microbe interactions have been immensely studied by multiomics approaches which have generated multilayered information about what is actually happening in real time within the cells (Meena et al. 2017). Microbial communities in polar and alpine region are vulnerable to different environmental stresses, using metagenomics. Varin and others analyzed the metagenomes of *Cyanobacteria*, which are the genes coding for functional responses to environmental stresses in the Arctic and Antarctic region, as well as studied the diverse mechanism of adaptation to cold and other stresses (Varin et al. 2011). Before the onset of the omics technology, genetic approach was termed as "candidate gene by gene" which involves identification and study of gene to know its signalling

cascade contributing to stress responses (Gupta et al. 2013). Plants have various adaption mechanisms during stress condition which involves physiological and biochemical changes resulting in adaptive morphological changes. Earlier genomic studies on plant stress responses due to environmental factor only gave a brief idea about the gene responsible for the phenotypic effect and its function (forward and reverse genetics, respectively). Recent studies deal with the post-genomic technologies by comprehensive analysis using functional genomic studies such as transcriptomics, proteomics, and metabolomics to enhance our understanding for the complex regulatory mechanism related to stress adaptation and tolerance (Urano et al. 2010).

Transcriptomics is the advancement of genomic studies which help us to know about the actively transcribed region of the DNA and to interpret the functionality of the genome. Transcriptional profiling by direct cDNA sequencing provides information about the resistance ability of a species with increasing climatic extreme predicted under global warming (Franssena et al. 2011). Research on plant proteomes has provided a beneficial information for comprehensive understanding of protein networks in plants in response to stimuli (Hashiguchi et al. 2010). Apart from the classical two-dimensional gel electrophoresis along with mass spectroscopy which has been widely used in proteomic study, the rise of the next-gen proteomic tools, such as stable isotope labeling with amino acids in cell culture (SILAC), isobaric tags for relative and absolute quantification (iTRAQ), multiple reaction monitoring (MRM), single reaction monitoring (SRM), sequential window acquisition of all theoretical fragment ion spectra (SWATH), and matrix-assisted laser desorption ionization (MALDI) imaging, has opened wide opportunities and high qualitative proteomic study (Langridge and Fleury 2011; Kumar and Kumari 2018).

Integrated "omics" analysis centered on metabolomics can be a powerful technique to identify the functions of genes involved in the metabolomics process. The majority of specialized metabolites induced by abiotic stress characteristically exhibits antioxidant activity in vitro, but their function in vivo is largely yet to be experimentally confirmed (Nakabayashi and Saito 2015). Even a complete understanding of the state of the genes, messages, and proteins in a living system does not reveal its phenotype. Metabolites are the main readouts of gene vs environment interactions and represent the sum of all the levels of regulations in between gene and enzyme. Therefore, metabolome can be considered as the final recipient of biological information flow (Amelia et al. 2018). To cope up with the environmental stresses, plants adapt some alternative mechanism to regulate its normal physiochemical mechanism. The heritability of reversible epigenetic modifications that regulate gene expression without changing DNA sequence makes them an attractive alternative mechanism (Boyko and Kovalchuk 2011).

12.2 Genomics in Agriculture

Although the effects of climate change are still hard to predict, the likely climate-related stressors for plants are cold, heat, drought, submergence, pathogens, and pests (Kole et al. 2015; Scheben et al. 2016). For characterization of a population,

next-gen sequencing has allowed researcher to explore around different population and persistence of population during climatic change providing local adaptation and phenotypic plasticity (Stillman and Armstrong 2015). One such example is metagenomics, where population of microorganism can be characterized by 16sRNA, which is a phylogenetic marker, used to test from permafrost-associated soil (Mackelprang et al. 2016). Genotyping by sequencing is being widely used in several species and is gaining more attention because of its cost-effectiveness. For genotyping, DNA marker technology has developed rapidly during the past few years. Apart from electrophoresis, molecular hybridization, and molecular marker based on PCR, in addition, there are molecular markers based on chip technology such as diversity array technology (DArT) and single-nucleotide polymorphism (SNP) which are technically important for genomic selection in crop breeding for multiple trait analysis, for which information of multiple SNPs are available in the bioinformatics database, e.g., *dbSNP* in NCBI, OMIM, and others (Deshmukh et al. 2014; Wang et al. 2018).

Attention given to methodologies like quantitative trait loci (QTL), genome-wide association studies (GWAS), etc. for comparative genomics and identification of potential genomic loci, genes, and biochemical pathways engaged with stress resilience has been well studied in wheat and rice (Shah et al. 2018).To find the correlation between the loci in the DNA and its phenotypic trait, QTL (quantitative trait loci) can be done for mapping such region on different population. QTL mapping was done for 104 rice varieties (*Oryza sativa L.*) of double haploid lines under drought stress by Tripathy and co-workers. In QTL mapping for CMS, it was found that composite interval mapping identified nine QTLs for CMS located on chromosome 1,3,7,8,9,11, and 12. Phenotypic variation was also explained by QTLs, ranging from 13.4% to 42.1% (Tripathy et al. 2000).

QTL study has also been done on barley for cold stress, to find the region of the genome linked to quantitative phenotypic trait. Skinner and his co-workers found 20 C-repeat binding factor (CBF) genes in barley which are the key regulators of cold tolerance genes using QTL (Skinner et al. 2006). For mapping of heat-tolerant genes and heat-associated genes, Langdon chromosome substitution lines were used for the first time and were found on chromosomes 3A, 3B, 4A, 4B, and 6A and reported that chromosome 3A,3B, and 3D were associated with heat tolerance in wheat cultivar (Sun and Quick 1991; Xu et al. 1996; Ni et al. 2018).

A review on stress genomics by Zhongfu Ni et al. was done for wheat varieties demonstrating that fine mapping techniques could be applied to identify genes on the chromosomal region associated with stress tolerance. It was analyzed by Acuna-Galindo et al. and found that by QTL meta-analysis, eight major QTL clusters were identified on chromosomes 1B, 2B, 2D, 4A, 5A, and 7A associated with drought and heat tolerance (Acuna-Galindo et al. 2015; Ni et al. 2018). Similarly, Islam et al. conducted QTL meta-analysis for salt tolerance in rice (Islam et al. 2019).

Genome-wide association study (GWAS) has high-resolution mapping capability and also explores a wide range of allelic diversity (Deshmukh et al. 2014). GWAS study was made in heat stress condition for *Brassica napus*. SNPs were able to identify 115 significant markers and 20 QTL linked to heat stress (Rahmana et al.

2018). Genome-wide association study was also done for wheat in 130 diverse elite line and landraces under heat stress and checked for the ethylene production for spike growth and studied the genotypic variation to identify the genetic loci (Valluru et al. 2017). Heat shock transcription factor (HSF) regulates the heat shock responses by regulating the expression of heat shock proteins during heat stress. In a comparative study made on rice and *Arabidopsis* under heat stress, complement of HSF genes has probably been identified through the genome-wide scan and also checked for their phylogenetic relation. It was found that three major cluster of orthologous genes belonging to both the species must be representing a common ancestor (Guo et al. 2008).

Nowadays to simplify genomic study, multi-environmental trial (MET) has come up for selection of genomic model, during particular stress condition with the help of biomarker. The use of factorial regression genomic best linear unbiased predictor (FR-gBLUP) is a way of genomic random regression to model a reaction norm to genotype during environmental stress condition which was performed in wheat. Genotype by environment (GxE) factor for FR-gBLUP was found to have performed better than an additive model, and it also provides an insight into the understanding of GxE and broadens the choice of genotypes that can be recommended to withstand particular environmental stress or used for adaptation breeding (Ly et al. 2018).

Such experiments give an idea about the genomic tools which could be broadly used in regard to gene knowledge to discover and tag individual alleles and to develop and deploy molecular markers to track the desired alleles in future breeding programs (Langridge and Reynolds 2015).

With the advent of CRISPR/Cas9 genome editing technology, the structure of abiotic stress tolerance in plants and other organism has paved the way in the field of genomics. There were very less liturature available on the abiotic stress using the genome editing technology however many studies available on biotic stress. Ri Li et al. studied for *SlNPR1* gene and generated *slnpr1* mutant induced by CRISPR/cas9 from tomato to study its involvement in drought stress response level. Results showed that slnpr1 mutant reduced the tolerance level and concluded that *SlNPR1* gene is involved in regulating drought responses (Rui Li et al. 2019). A customized toolkit CRISPR/Cas9 has been widely used in *Arabidopsis* knockout mutant studies. The cold-inducible C-repeats/DRE-binding factors like CBF1, CBF2, and CBF3 have been exercised to generate mutant lines by CRISPR/Cas9 (Cho et al. 2017; Debbarma et al. 2019). CRISPR editing has been carried out recently on two novel genes *GhRDL1* and *GhRDL1–3* in cotton to combat drought stress. Similarly in cassava crop, MeKUPs, which responds to multiple abiotic stresses like osmosis, salt, drought, and cold, were addressed using CRISPR/Cas9 system. Genome engineering is also been done in banana MaAPL3 under cold and salt stress (Dass et al. 2017; Miao et al. 2017; Haque et al. 2018; Ou et al. 2018; Debbarma et al. 2019). In rice also, a salt-sensitive *OsERF922* gene and a drought-responsive gene *OsDREB* have been targeted using CRISPR/Cas9 nuclease (Liu et al. 2012; My et al. 2016; Debbarma et al. 2019). Thus, this tool has validated and raised the bar for better performance than the traditional genetic crosses.

12.3 Transcriptomics

Transcriptomics refers to the study of complete set of RNAs (transcriptome) encoded by the genome of a specific cell or organism at a specific cell or organism at a specific time or (transcriptome) encoded by the genome of a specific cell under a specific set of conditions. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues and also for understanding development and disease. The key aims of transcriptomics are to catalogue all species of transcript, including mRNAs, noncoding RNAs, and small RNAs; to determine the transcriptional structure of genes, in terms of their start sites, 5′ and 3′ ends, splicing patterns, and other posttranscriptional modifications; and to quantify the changing expression levels of each transcript during development and under different conditions.

Microarrays quantify a set of predetermined sequences and are frequently used to assess comprehensively the relative or absolute abundance of individual RNA transcripts. Microarray technology provides a means of studying multiple pathways and mechanisms at the same time (Aardema and Grego 2002). This technique has provided a broad impression of how organisms respond to environmental stressors and the possibility to identify novel ecotoxicological biomarkers (Sevillano et al. 2014). Todgham and Hoffman (2009) carried out microarray-based transcriptomic analysis of the physiological response of larvae of a calcifying marine invertebrate, the purple sea urchin, *Strongylocentrotus purpuratus*, to CO2-driven seawater acidification. According to them, genomic-based studies have the potential to identify potential "weak links" in physiological function that may ultimately determine an organism's capacity to tolerate future ocean conditions.

RNA-Seq is a recently developed approach to transcriptome profiling that uses deep-sequencing technologies (Wang et al. 2009). RNA sequencing (RNA-Seq) provides certain advantages over DNA oligonucleotide microarrays, including broader transcriptome coverage with the detection of rare or novel transcripts, alternatively spliced forms, and allele-specific expression. In addition, RNA-Seq can provide better quantitation over a broader dynamic range with reduced noise, enabling more subtle changes to be quantitated reliably. RNA-sequencing method has already been applied to *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, mouse, and human cells. Using RNA-sequencing technology, May et al. 2013 demonstrated that both CO2 and temperature alter the microRNA expression to affect *Arabidopsis* growth and development and that miR156/157- and miR172-regulated transcriptional network might underlie the onset of early flowering induced by increasing CO2. Recently the direct RNA sequencing (direct RNA-seq) using nanopore arrays has been used for characterizing herpes simplex virus type 1(HSV-1) transcriptome (Depledge et al. 2019).

Transcriptome analysis has helped identify significant alteration of gene expression associated with the inflammatory or immune response, the cholesterol efflux, and the adrenocortical zonation in the adrenocortical cells of Star2/2 mice. The transcriptome in contrast to the genome is highly variable over time between cell types and will change in response to environmental changes. Gene expression

profiling can be used to determine which genes are differently expressed as a result of changes in environmental conditions (Vlaanderen et al. 2010). The impact of metals on cellular metabolism and global homeostasis has been traditionally assessed in free-living organisms by using conventional biomarkers such as free-living mice *Mus spretus*, a conventional bioindicator used to monitor metal pollution (Sevillano et al. 2014). Transcriptomic tools aided the study of various constituent members of a subgroup of algae that are responsible for forming harmful algal blooms (HABs) and also their genesis and function (Mclean 2013).

ChIP-chip technology, the chromatin immunoprecipitation (ChIP) procedure combined with transcriptomics technology, can also be used to investigate the genome-wide location and function of DNA-binding proteins (Zhang et al. 2010). This was initially established in yeast (Ren et al. 2000). Transcriptomics has revolutionized our understanding of how genomes are expressed. Also integration with other omic technologies is giving an increasingly integrated view of the complexities of cellular life.

12.4 Proteomics

Proteins are ultimately functional molecules involved in most cellular processes. Since proteins serve as important components of major signalling and biochemical pathways, studies at protein levels are essential to reveal molecular mechanisms underlying plant growth, development, and interactions with the environment (Chen and Harmon 2006). Proteomics is the large-scale study of proteomes. Proteomes are a set of proteins produced in an organism, system, or biological context. The term proteomics was first coined by James to make analogy with genomics, the study of genes, and is often seen as next step in the study of biological systems, after genomics (Wilkins et al. 1997). In contrast to genome, the proteome is highly variable over time, between cell types, and will change in response to changes in its environment. Proteomics complements other functional genomics approaches, including microarray-based expression profiles 2, systematic phenotypic profiles at the cell and organism level, systematic genetics, and small-molecule-based arrays (Tyers and Mann 2003). Proteomic-based toxicity studies and biomarkers are highly relevant to biological functions, adverse health outcomes, and health risk assessments (Ge et al. 2013). The changes occurring in the microorganism exposed to anthropogenic pollutants were determined with the help of proteomic tools (Kim et al. 2004). Proteomic approaches can help reveal the toxicity mechanism by identifying the proteins that are altered after pollution exposure in an organism (Sevillano et al. 2014).

The commonly used analytical proteomic techniques are mass spectrometry (MS)-based approaches and nuclear magnetic resonance (NMR) spectroscopy, although others exist, including quantitative/comparative proteomics (2D-PAGE, ICAT, SILAC, and iTRAQ), array-based technologies (antibody arrays, protein lysate arrays, peptide arrays, aptamer arrays, and bead-based arrays), and the multi-epitope-ligand cartography (MELC) technology (Singh et al. 2010).

Davies (2010) characterized three main techniques that are used for quantitation in proteomics:

1. Two-dimensional polyacrylamide gel electrophoresis (2D- PAGE) linked to mass spectrometry for protein identification
2. Stable isotope labeling
3. Stable isotope label-free shotgun proteomics

Several PAH-induced proteins were identified in *Mycobacterium vanbaalenii* PYR-1 grown in presence of high-molecular-weight polycyclic aromatic hydrocarbons (HMW-PAHs) using two-dimensional gel electrophoresis (2-DE) (Kim et al. 2004). The analysis of the complete proteome of *Arabidopsis* cells was attempted by Giavalisco et al. (2005) using the 2D-PAGE and MALDI-TOF peptide mass fingerprinting. Kim et al. (2006) analyzed aromatic hydrocarbon catabolic pathways in *Pseudomonas putida* KT 2440 using a combined proteomic approach based on 2DE/MS and cleavable isotope-coded affinity tag analysis.

Tandem mass tag (TMT) quantitative proteomics and targeted metabolomics approaches have provided important insights into the JAZ7-regulated molecular networks of drought tolerance (Meng et al. 2019). They found 394 unique proteins and 96 metabolites were enriched in JA and abscisic acid (ABA) signalling pathways and responded to stress, photosynthesis, redox, and metabolic process. The knowledge may facilitate effort to enhance crop drought tolerance in the era of climate change. An understanding of the growth conditions governing the expression of the proteome in a specific environment is essential for developing rational strategies for successful bioremediation. Many proteomic tools like 2-DE have helped in understanding the mechanisms employed by bacteria to degrade aromatic hydrocarbons and the associated physiological responses.

The activated sludge carrying microorganisms embedded in extracellular polymers (biofilms) is highly efficient in removing zinc, cadmium, and nickel from aqueous solutions from a combined activated sludge (AS)-biofilm process (Chang et al. 2006). *Nannochloropsis oceanica* is a large group of photoautotrophic eukaryotic organisms that play important roles in fixation and cycling of atmospheric CO2. Its capability of storing solar energy and carbon dioxide in the form of triacylglycerol (TAG) of up to 60% of total weight under nitrogen deprivation stress sparked interest in its use for biofuel production. Chen et al. (2019) combined proteomic and fluorescence-activated cell sorting (FACS) for identifying the molecular principles of single-cell-level phenotypic heterogeneity in lipid storage of *Nannochloropsis oceanica*.

It is impossible to understand mechanisms of disease, ageing, etc. solely by studying the genome. Only by understanding protein function and their modifications, drug targets for various diseases can be identified. Proteomics provides a powerful set of tools for the large-scale study of gene function directly at the protein level. In particular, the mass spectrometric study of gel-separated proteins is leading to a renaissance in biochemical approaches to protein function (Pandey and Mann 2000).

12.5 Metabolomics

The term metabolomics is defined as comprehensive and quantitative analysis of all small molecules in a biological system (Fiehn O 2001; Obata and Fernie 2012). Kaplan et al. (2004) explore the mechanisms of plant adaptation to thermal stress at the metabolite level, reveal relationships between heat- and cold-shock responses, and highlight the roles of known signaling molecules and protectants. They identified 81 identified metabolites and 416 unidentified mass spectral tags, characterized by retention time indices and specific mass fragments, and reported that cold shock influenced metabolism far more profoundly than heat shock. The steady-state pool sizes of 143 and 311 metabolites or mass spectral tags were altered in response to heat and cold shock, respectively. To understand the biology and development of stress-tolerant plants, the use of mass spectrometry (MS)-based analytical platforms to profile the stress-responsive metabolome has greatly helped to see through the reason behind adaptation of plants to adverse environmental condition which is fundamental to current plant biotechnology research programs (Jorge et al. 2016).

Compounds that inhibit formation of free radicals are called antioxidant. During environmental stress condition, metabolites produced by the plants exhibit in vitro antioxidant activity. Plants under stress condition usually limit the uptake of CO_2 which results in the production of hydrogen peroxide (H_2O_2), superoxide(O^{2-}), and singlet oxygen ($^1O_2$) radicals as a result of over-reduction of the photosynthetic electron transport chain leading to production of ROS (reactive oxygen species) (Benina et al. 2013; Noctor et al. 2014; Hossain et al. 2015; Esfahani et al. 2016; Abdelrahmana et al. 2017). In *Arabidopsis*, a network of at least 152 genes is involved in managing the level of ROS. This network is dynamic and redundant and encodes ROS-scavenging and ROS-producing proteins (Mittler et al. 2004). Abscisic acid (ABA) is variously known to produce under drought responses and is essential to response under stress condition. Phytoharmone plays an important role in closing of stomata, and in response to several enzymes for ABA, biosynthesis and catabolism have been identified (Seki et al. 2007).

Proline, an amino acid, is the most widely distributed osmolyte which is produced during different environment stress conditions (Verbruggen and Hermans 2008; Szabados and Savoure 2010; Rodziewicz et al. 2014) which was confirmed in transgenic plant by P5CS overexpression in tobacco, which counteracts for stress tolerance in plants (Kavi Kishor et al. 1995; Rodziewicz et al. 2014).

Literature survey has suggested that, during drought condition, plants tend to produce raffinose family oligosaccharide (RFO) sugars such as raffinose, stachyose, and galactinol which play important role to tolerate against dry and water-deficient condition and function as osmoprotectant during drought stress. In transgenic *Arabidopsis*, overexpression of galactinol synthase gene (AtGolS2) enhanced drought tolerance because of the accumulation of galactinol and raffinose. Transgenic plants that overexpress DREB1A/C-repeat binding factor 3 (CBF3) are tolerant of drought and cold stress and accumulate more galactinol and raffinose than wild-type plants (Taji et al. 2002; Avonce et al. 2004; Valliyodan and Nguyen 2006; Seki et al. 2007).

In response to dehydration stress, experiment has been conducted on *Arabidopsis thaliana* and found some dynamic metabolic network in response to dehydration stress. Urano and others did the metabolomics analysis for the same and characterized the metabolic phenotypes of *Arabidopsis* wild-type and a knockout mutant of the NCED3 gene (nc3-2) under dehydration stress, which play a role in dehydration-inducible biosynthesis of ABA. Metabolomics profiling using mass spectrometry system revealed that accumulation of amino acids depended on ABA production, and the oligosaccharide raffinose level was also regulated by ABA. An integrated metabolome and transcriptomics revealed ABA-dependent transcriptional regulation of the biosynthesis of the branched-chain amino acids, saccharopine, proline, and polyamine (Urano et al. 2009).

In *Pinus radiata* Escandon and his co-worker did the study in response to induced high temperature and saw dynamic changes in the metabolomes showing complex metabolic pathway interaction network related to heat stress. Using mass spectrometry techniques (GC-MS and LC-Orbitrap-MS), 2287 metabolites were quantified, and cytokinin, fatty acid metabolism and flavonoid, and terpenoid biosynthesis were the most important pathways involved in heat response with hormones like zeatin riboside (ZR) and isopentyl adenosine (iPA) that are involved in coordinating in such multiple complex interaction. Thermotolerance metabolic biomarkers like L-phenylalanine, hexadecenoic acid, and dihydromyricetin were crucial metabolites found in metabolic pathways as a strategy to adapt during heat tolerance (Escandon et al. 2018).

In chickpeas (*Cicer arietinum*) to identify the metabolite, a global metabolomics method, UPLC-MS, was used during drought stress. Twenty known metabolites were identified, such as proline, L-arginine, L-histidine, L-isoleucine, and tryptophan, and showed increased level in the tolerant line after drought stress induction, but decreased level of putrescine, choline, phenylalanine, gamma-aminobutyric acid (GABA), and alpha-ketoglutaric acid was unusual and could be used as biomarker in the future for abiotic stress studies (Khan et al. 2019).

12.6 Epigenomics

Epigenomics is the study of all the epigenetic modifications for an entire genome (Beck et al. 1999). Epigenomics is mainly based on two most comprehensively studied mechanisms, DNA methylation and histone modification. However, in recent years, RNA interference of gene expression by noncoding RNAs such as microRNA and siRNA has acquired considerable attention (Vlaanderen 2010). Molecular analysis, especially through omics approaches, of these primary lines of environmental adjustment in the context of climate change has revealed the underlying biochemical and physiological mechanisms, thus characterizing the links between phenotypic plasticity and climate change responses (Bigot et al. 2018). Epigenetic mechanisms appear to allow an organism to respond to the environment

through changes in gene expression (Jaenisch and Bird 2003), and environmental conditions have also been reported to affect levels of methylation (Finnegan et al. 1998, 2000). Du et al. 2019 suggested that the nature of chromosomal rearrangement in cancer is related to the spatial and temporal positioning and altered epigenetic states of early-replicating compared to late-replicating loci. Methylation is the most common flexible genomic parameter that can change genome function under exogenous influence and usually occurs in CpG islands, a CG rich region, in the DNA (e.g., promoter regions, regulatory domains, and also in intergenic regions). A number of studies have described DNA hypomethylation in several tumor types, such as colorectal and gastric cancers and melanomas. Another important epigenetic alteration is histone modification in cancer cells, and it may affect the gene transcription through local relaxation of nucleosomal structure and through recruitment of nonhistone proteins, which can be chemically modified by different enzymes at their external N- and C-terminal tails as well as at internal histone-fold domains (Yan et al. 2015). Gac et al. analyzed the possible involvement of epigenetic mechanisms in the winter-dormant shoot apical meristem of Populus × euramericana clones in memory of the growing conditions faced during the vegetative period.

Recent technical advances, such as whole-genome bisulfite sequencing and affordable epigenomic array-based technologies, allow researchers to measure epigenetic profiles of large cohorts at a genome-wide level, generating comprehensive high-dimensional datasets that may contain important information for disease development and treatment opportunities.

The epigenetic augmentation of species evolutionary potential (its regulation through gene expression) can enable K-strategists to survive and adapt to different environments, and this mechanism may be particularly important for the persistence of sharks, skates, and rays in the light of future climate change (Lighten et al. 2016). Epigenetic mechanisms directly regulate genetic processes and can be dramatically altered by environmental factors. Therefore, environmental epigenetics provides a molecular mechanism to directly alter phenotypic variation generationally (Skinner 2015). Recent findings suggest that quercetin and other flavonoids may possess the capacity to counteract the adverse epigenetic regulation involved in various forms of cancer (Russ and Ungaro 2019).

There is increasing evidence for the involvement of epigenetics in human disease such as cancer, inflammatory disease, cardiorenal syndrome, and CV disease. Other chronic diseases are also susceptible to epigenetic modification such as metabolic diseases including obesity, metabolic syndrome, and diabetes mellitus (Bihaqi 2019; Bhagirath et al. 2019; Stylianou 2013, 2019; Virzi et al. 2017; Whayne 2014).

Epigenetic changes that control phenotypes might be used as markers for monitoring climate change and subjected to genetic engineering to improve the plant traits permanently. Thus, understanding how plant epigenomes respond to climate change and if such changes are heritable will enable better predictions about how climate change will affect plants (Liu 2013).

12.7 Current Status and Future Prospects

Extreme environmental changes and fluctuations mainly derived by climate changes have profound effect on organism (Garcia-Cela et al. 2018). Genomics, transcriptomics, proteomics, metabolomics, and epigenomics are some of the omics approaches which have been helpful to make out studies in the changes that have occurred due to stress condition.

Traditionally, genes have been analyzed individually, but microarray technology has advanced substantially in recent years. DNA microarrays measure differences in DNA sequence between individuals, and the expression of thousands of genes can be analyzed simultaneously (Horgan and Kenny 2011). Keeping in view the relevance in the context, large-scale genomic tools particularly based on NGS technologies have emerged as potential addition to conventional breeding. Recent advancement in the omic platforms has been eased and couldn't be possible to promote research activities without the utilization of the available computational resources that helps to catalogue, store, and make it easily accessible through databases (Deshmukh et al. 2014). CRISPR/Cas9 is widely used in biotic stress tolerance for plants, and it may also useful for the abiotic stress tolerance, and production of transgenic lines using the technology strategically. Plants use multiple strategies during differential environmental conditions leading to great variability in expression of different stress-induced proteins, namely, HSPs, AFPs, RBP, and LEA, and detoxification enzymes which likely contribute to plant adaptation. Complex studies of omics under variable stress condition will be required for better understanding of signalling pathways (Kumar and Kumari 2018). However, metabolomics studies have greatly eased in mapping out the pathways by knowing the metabolomes involved during stress condition. Therefore, high-throughput data analysis and integrated omics approaches will generate a thorough insight into the climate-responsive changes in an organism.

12.8 Conclusion

The potential applications of the omics tool are crucial to understand the whole processes of molecular networks in response to abiotic stress. To combat against any stress condition, plant tries to adapt where changes occur genotypically and phenotypically. Combining omics tools with breeding activities incorporates stress tolerance in plant due to climatic change. The advancement of biotechnological tools such as genetic/metabolic engineering and marker-assisted breeding along with the combination of omics approaches has shown great potential for production of abiotic stress-tolerant crops. Climate change is an exponential factor; thus such stipulation demands a smarter way to produce climate-resilient crop.

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13 Plant and Microbial Genomics in Crop Improvement

Indu Rialch, Saurabh Singh, Rajender Singh, and Arun Kumar

Abstract

Classical soil science approaches have enabled us to establish basic principles of how the soil system functions and have answered numerous practical agricultural application questions. In recent years, efforts have been refocused on better understanding, managing, and benefiting from this system that contains one of the most complex biological communities of the planet. Soil biology is seen as being at the center of scientific research of this century with novel research objectives and goals being set. Genomics and metagenomics along with microbiological techniques are contributing greatly to advances in our understanding of living systems that exist in the soil and their interaction with plants. For its part, molecular plant nutrition has made significant progress in understanding the use of nutrients by plant cells and has identified molecular mechanisms that can improve nutrient use efficiency. Together, molecular soil microbiology and molecular plant nutrition are projected to be a driving force in agriculture and sustainable food production in the coming years.

Keywords

Plant genomics · Microbial genomics · Crop improvement

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13.1 Introduction

The world population by the end of 2050 is estimated to touch the figure of ten billion according to United Nations Department of Economic and Social Affairs. Thus, we are confronting the challenge to produce sufficient food with limited resources in sustainable manner to feed the consistently increasing population. Over the past decades, plethora of biotic stresses have emerged as crucial player in hampering the agricultural production (Singh et al. 2018; War et al. 2018). The crop plants rely on innate immunity to defend themselves against pathogens and insect herbivores, but the fight to combat attack of plant pathogens depends upon how rapidly and strongly the innate immune system gets activated. To avoid the excessive use of agrochemicals, the genetically modified (GM) crops have been used across the world (Shukla et al. 2018). But this approach has faced serious criticism and conflict between GM scientists and farmers, consumers, and environmentalists regarding human health, risk to food security, environment health, loss of biodiversity, generation of superweeds, enhancement in antibiotic resistance, allergic problems, and other inadvertent effects (Maghari and Ardekani 2011; Zhang et al. 2016). Therefore, there is a need of eco-friendly management strategies, and it urged to invest and research more to explore the role of plant-associated microbiome in crop production and protection. The important soil microbes associated with plants are classified as fungi, bacteria, algae, protozoa, and actinomycetes. The rhizosphere, which is the zonal soil area around plant root system, is considered as microbial hot spot as this area is enriched with high concentration of nutrients and accordingly rich diversity of microorganisms. Diverse array of interactions is exhibited between plant and these soil-dwelling microbes which have significant role in plant growth and root development (Jacoby et al. 2017). The root colonization by beneficial microbes in rhizosphere may result in induced systemic resistance (ISR) to defend plants against attack of pathogens (Sachdev and Singh 2018; Mhlongo et al. 2018). The well-known plant-microbe interactions are between plant and arbuscular mycorrhizal (AM) fungi, legume crops, and nitrogen-fixing bacteria, all of which have contribution in nutrient cycling and enhancing nutrient uptake capacity for sustainable crop production (Chen et al. 2018). The microbial diversity is the key factor to combat different plant diseases and enhancing crop production. Various microbial agents have been identified to control plant pathogens in different crops; some of the examples have been cited in Table 13.1. The microbes playing a role as biofertilizers in promoting crop production are also termed as plant growth-promoting rhizobacteria (PGPR), which mainly stimulate the plant root colonization and elicit plant growth along with reduction in incidence of harmful pathogens (Hass and Defago 2005). Over the past few decades, multiple modern genomics-assisted technologies and availability of whole genome sequencing of different plants and microbes have facilitated the downstream understanding of beneficial plant-microbe interaction and its potential in crop improvement.

Table 13.1 Some of important microbial agent in crop protection

Microbial agent	Target pathogen	Crop example	Reference (s)
Pseudomonas fluorescens, *Pseudomonas species*	*Pythium ultimum*, *Xanthomonas campestris*, *Fusarium oxysporum*, *F. moniliformae*, *R. solani*	Pea, tomato, potato, lettuce, cauliflower, cucumber	Hass and Defago (2005)
Pseudomonas fluorescens	*Colletotrichum lindemuthianum*	*Phaseolus vulgaris*	Ganeshan and Kumar (2005)
Pseudomonas fluorescens	*Pythium aphanidermatum*	Turmeric	Prabhukarthikeyan et al. (2018)
Bacillus subtilis	*Pythium ultimum*	Apple	Meena and Kanwar (2015)
Bacillus subtilis	*Podosphaera fusca*	Cucurbits	Meena and Kanwar (2015)
Bacillus subtilis	*Phomopsis phaseoli*, *Botrytis cinerea*, and *Bremia lactucae*	Tomato, lettuce	Etchegaray et al. (2008)
Bacillus subtilis	*Fusarium oxysporum*	Cucumber	Cao et al. (2012)
Bacillus subtilis	*Fusarium culmorum*	Wheat	Falardeau et al. (2013)
Bacillus circulans	*F. oxysporum f. sp. lycopersici*	Tomato	Hsieh et al. (2008)
Bacillus pumilus	*Rhizoctonia solani*, *Pythium aphanidermatum*, and *Sclerotium rolfsii*	Tomato, watermelon, cowpea, sugar beet, rice, wheat	de Melo et al. (2009)
Bacillus amyloliquefaciens	*Rhizoctonia solani*	Soybean	Chowdhury et al. (2015)
Bacillus amyloliquefaciens	*Agrobacterium tumefaciens*	Carrot	Ben Abdallah et al. (2015)
Trichoderma virens	*Pythium ultimum*, *Pythium aphanidermatum*, *Fusarium oxysporum*, *Rhizopus oryzae*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Pythium* spp.	Tomato, chilli, potato, rice	Muthukumar et al. (2011)
Lactobacillus plantarum	*Xanthomonas arboricola* pv. *pruni*, *Pseudomonas syringae* pv. *actinidiae*, *Xanthomonas fragariae*	Kiwi, strawberry, *Prunus*	Daranas et al. (2019)

13.2 Genomics of Nitrogen-Fixing Bacteria-Legume Symbiosis

The nitrogen sources, like nitrate and ammonia, which are potential regulator of plant growth and development, are frequently becoming limited. The plant species belonging to nitrogen-fixing clade have the potential to combat this limitation. The presence of root nodules in these plant species leads to symbiotic relationship with nitrogen-fixing bacteria (MacLean et al. 2007). These bacteria mainly comprise the members of genera *Rhizobium*, *Azorhizobium*, *Sinorhizobium*, *Mesorhizobium*, and

Bradyrhizobium (MacLean et al. 2007). The other classes of β-proteobacteria related to *Burkholderia* and *Cupriavidus* genera isolated mainly from *Mimosa* species or papilionoid legumes are also capable to fix nitrogen by nodulating legumes (Liu et al. 2012; Lardi and Pessi 2018). This symbiotic biological nitrogen fixation process in agricultural systems has enormous potential for sustainable crop production avoiding harmful chemical fertilizers and is a paradigm in legume-microbe signaling.

The availability of whole genomes of rhizobia, viz., *Rhizobium leguminosarum* bv. *viciae* (Young et al. 2006), *Rhizobium sullae* type strain IS123^T (Sablok et al. 2017), *Rhizobium leguminosarum* Norway (Liang et al. 2018), *Rhizobium jaguaris* CCGE525^T (Servin-Garciduenas et al. 2019), *B. japonicum* (Kaneko et al. 2002), *Mesorhizobium loti* (Kaneko et al. 2002), *S. meliloti* (Barnett et al. 2001; Galibert et al. 2001), and two photosynthetic *Bradyrhizobium* strains (Giraud et al. 2007), has allowed elucidation of specific features of each rhizobium, their molecular dialogue with respective plant host species, identification, and characterization of series of symbiotic genes. The considerable variation is present in genomic architecture of these species (MacLean et al. 2007).

The symbiotic genes relevant for nitrogen fixing are commonly clustered within the symbiosis islands (Sis) or on large plasmids (pSym) emphasizing the supplementary role and nature of the genes. The presence of different insertion sequence (InSeq) elements, transposable elements (TE), and related genes, within regions encoding symbiotic functions, makes rhizobial genomes highly dynamic in nature (MacLean et al. 2007). The genome plasticity studies in rhizobia have demonstrated the role of extensive recombination and presence of repeated DNA sequences and multiple replicons in dynamic and instable nature of rhizobial genome (MacLean et al. 2007). Some of the nodule bacteria such as *Bradyrhizobium* sp. strains BTAi1 and ORS278 (Giraud et al. 2007) have high metabolic and ecological diversity and highly complex genomes. The genome sequence analysis of *Rhizobium leguminosarum* indicated more common genes share with *S. meliloti* and *M. loti* in contrast to its closely related phytopathogen *Agrobacterium tumefaciens* (Young et al. 2006).

13.3 Plasmidomics

The genomes of rhizobium bacteria commonly consisted of a chromosome and different plasmids varied from 150 Kb to 1800 Kb sizes. The plasmid regions have been reported to account for 40% of the total genomic content harboring genetic information inducing symbiosis (MacLean et al. 2007*). As in many of rhizobia, the genes governing nodulation and nitrogen fixation are clustered in plasmid (plasmid borne), the sequencing of various rhizobial plasmids carried out prior to whole genome sequencing projects. In this context the first attempt was complete sequencing of symbiotic plasmid of Rhizobium sp. pNGR234a* (Freiberg et al. 1997*) and revealed high frequency of InSeq and related sequences in the rhizobial genomes. Then, the sequencing of symbiotic plasmid of Rhizobium etli CFN42 revealed the mosaic structure and other symbiotic regions of rhizobia. The sequencing of the nonsymbiotic plasmids, such as* 144 kb pSmeSM11a from *S. meliloti* (Stiens et al.

2006), *revealed their role in overall microbial fitness. This nonsymbiotic plasmid harbors the genes encoding proteins which induce DNA recombination, replication, and repair, as well as other metabolic enzymes and transport systems.*

13.3.1 Functional Genomics of Legume-Bacteria Symbiosis

13.3.1.1 Transcriptomics

The transcriptome is the complete set of RNA transcripts in a cell and their quantity and comprised of coding (mRNA) and noncoding RNAs (Srivastava et al. 2019). Transcriptomics has enabled the investigation of change in expression level of genes encoded in a genome in response to change in environment or other defined developmental changes. The major functions of transcriptomics comprise indexing of all the species of transcript, constituting coding and noncoding RNAs and sRNAs (small RNAs), and then determining gene's transcriptional structure, transcriptional modifications, and quantification of change in expression level (Srivastava et al. 2019). The main contemporary approaches for transcriptome analysis consisted of DNA microarrays and RNA-seq (RNA-sequencing). Different reports are available regarding transcriptome profiling of rhizobia in response to perceiving single flavonoid under free living environment (Lardi and Pessi 2018). Studies have been conducted regarding change in transcriptome in the presence of root exudates (RE) from host and nonhost legume plants and also nonlegume plants (Lardi and Pessi 2018). Comparative transcriptomic analysis of *Rhizobium leguminosarum* biovar *viciae* 3841 in the rhizosphere of pea, *Medicago sativa*, and *Beta vulgaris* revealed the common set of genes such as *dctA* inducing C_4-dicarboxylate transport and *rmrA* encoding efflux pump (Ramachandran et al. 2011). Recently, Liu et al. (2012) employing transcriptomics techniques and analyzed the effects of *Glycine max* root exudates on two strains 4534 and 4222 of *B. diazoefficiens* and reported various genes encoding for two-component systems (nodW, phyR-*s*EcfG); bacterial chemotaxis (cheA), ATP-binding cassette (ABC) transport proteins, and indole-3-acetic acid (IAA) metabolism were upregulated in the more competitive *B. diazoefficiens* strain 4534. Genome-wide macroarray experiments performed upon bacteroids from *M. loti* strain MAFF303099 resulted in the identification of expression islands scattered across the symbiotic island (Uchiumi et al. 2004).

13.4 Proteomics in Plant-Microbe Interaction

Knowledge of protein expression is essential for understanding biological processes. Proteomics includes large-scale analysis of proteins. Protein phosphorylation and degradation are also known to occur during plant defense signaling cascades. Proteomic technology is based on the combination of two-dimensional electrophoresis (2-DE), allowing the separation of denatured protein polypeptides according to their isoelectric points and molecular weights, and mass spectrometry (MS) identification methods, either by peptide mass fingerprinting or de novo

sequencing. The use of proteomic analysis, based on two-dimensional electrophoresis followed by mass spectrometry, is to characterize symbiosis-related proteins, and it is also possible to gain greater insight into the detailed impact that plants and soil microbes especially bacteria have with each other. Mass spectrometry (MS) is a very robust tool in large-scale proteomics especially coupled with electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) which can transform macromolecules to ions in the gas phase for mass spectrometric analyses without losing their structure or form. These differently modified forms of protein execute vital cellular functions including a plant perception and defense against pathogens during plant-bacterial interactions. Using similar technique by Kwon et al. (2016), successfully analyzed total of 41 differentially expressed proteins in response to *Paenibacillus polymyxa* and with the help of molecular function-based bioinformatics tools resulted in their classification into 7 different proteins groups. MS-based proteomics has also been applied in an attempt to detect the presence of phytopathogens in plant-bacterial interaction studies.

Plant and microbes can interact with each other in a variety of different ways. Plants secrete various organic compounds resulting in a nutritionally enriched environment favorable for microbial growth. As a result, plants are heavily colonized with a diversity of microbes especially fungi and bacteria, whose reservoir is primary the soil. The interaction among plant and microbes may be beneficial, harmful, or neutral for the plant, or sometimes the impact of microbes may depend upon the conditions of the soil. While a number of different soil bacteria are phytopathogenic, the majority of the more agronomically important plant disease causing soil microorganisms is fungi (Table 13.2). Proteomic characterizations enable researchers to investigate the detailed response of plants and bacteria to various treatments and to one another. Most of the reported studies have focused on the detection of protein expression changes in response to toxicity, nutrient changes, mutations, or overexpression of particular genes. Proteomic studies of plant-bacterial interactions, the symbiotic interactions between nitrogen-fixing bacteria and legumes, have studied in the greatest details plant-pathogen interaction. Proteomics are also included in the studies of endophytic and rhizosphere plant growth-promoting bacteria (PGPB), likely in part because these organisms and their mode of action are less well characterized.

13.4.1 Direct Molecular Analysis of Soil Microbes

13.4.1.1 Nucleic Acid Hybridization

It involves the bonding of a short, complementary nucleic acid strand to a target sequence. The probe is generally labelled with a radioisotope or fluorescent molecule, and the target sequence is typically bound to a nylon membrane or other solid surface. This hybridization technique was used to analyze clinically important microorganisms. Nucleic acid hybridization probes are used to detect specific phylogenetic groups of bacteria, archaea, or eucarya in appropriately prepared soil

Table 13.2 A few specialized examples of plant-microbe interaction

Plant-microbe interaction	Host plant	Proteomic technique	Principal findings and references
Plant-microbe symbiotic interaction			
Paenibacillus polymyxa	*Arabidopsis thaliana*	2-DE approach in conjunction with MALDI-TOF/TOF	Analysis revealed a total of 41 differentially expressed proteins in response to *P. polymyxa* (Kwon et al. 2016)
Rhizobium leguminosarum	*Pisum sativum* and *Vicia cracca*	Microarray	Genes expressed related to tricarboxylic acid cycle, succinate, pyruvate/inositol catabolism, etc. (Karunakaran et al. 2009)
Bradyrhizobium japonicum	*Glycine max*	LCMS/MS, LTQ-Orbitrap MS	Proteins involved in translation, posttranscriptional regulation, nitrogenase complex, etc. (Delmotte et al. 2010)
Plant-microbe pathogenic interaction			
Fusarium graminearum	Wheat (*Triticum aestivum*)	2-DE, LC-MS/MS	Identified 15 induced proteins from wheat spikelets infected by *F. graminearum* (Zhou et al. 2005)
Fusarium verticillioides	Maize	2-DE, MALDI-TOF MS and nESI-IT MS/MS	Identified changes in protein patterns in germinating maize embryos in response to infection with the fungus *F. verticillioides* (Campo et al. 2004)
Puccinia triticina	Wheat	2-DE/Qq-TOF MS/MS	Identified 32 upregulated proteins from wheat leaves inoculated with *Puccinia triticina* (Rampitsch et al. 2006)
Erysiphe pisi	Pea	2-DE, MALDI-TOF MS	Identified leaf proteins implicated in powdery mildew resistance (Curto et al. 2006)
Cladosporium fulvum	Tomato	2-DE, MALDI-TOF MS LC-QTOF MS/MS	Identified three novel fungal secretory proteins, viz., CfPhiA, Ecp6 and 7 (Bolton et al. 2008)
Xanthomonas campestris	*Brassica oleracea*	2-DE, MALDI-TOF/TOF	Proteins from young leaves of susceptible *Brassica* cv infiltrated with *X. campestris* (Andrade et al. 2008)

samples by use of the FISH technique. The key advantage of FISH is the ability to visualize and identify organisms in their natural environment on a microscale.

13.4.1.2 Confocal Microscopy

Confocal laser scanning microscopy (CLSM), combined with in situ hybridization techniques, has been applied with considerable success to visualize the structure of soil microbial communities. The basic principle of CLSM is to create an image that is composed only of emitted fluorescence signals from a single plane of focus, giving insight into the 3D spatial relationships between microbial communities. This

technique was first used to observed interactions between wheat roots and *Azospirillum brasilense* SP7, a plant growth-promoting rhizobacterium (PGPR). Later association between bacterial beneficial strains and fungal in the root of sugar beet was studied by combining isosurface imaging and volume rendering to show the interactions between plant roots and fungal hyphae.

13.4.1.3 Biosensor and Marker Gene Technologies

Marker genes, such as luxAB (luminescence), lacZ (β-galactosidase), and xylE (catechol 2,3-dioxygenase), have been used successfully in soil microbial ecology studies. One such gene that has attracted a lot of attention in rhizosphere studies is gfp, which encodes the green fluorescent protein (GFP). This bioluminescent genetic marker can be used to identify, track, and count specific organisms into which the gene has been cloned and that have been reintroduced into the environment. Using this method some worker monitored the quantitation and activity of particular organisms specially bacteria, by gfp labelling to cells of *Rhizobium meliloti* MB5OI through pTB93F gfp plasmid.

13.4.1.4 Microarray

It is a powerful technique used to compare differences in gene expression between two or more nucleic acid samples. In this method, sample nucleic acids are probed, rather than the probes themselves, and are fluorescently labelled in contrast to other hybridization techniques. Labelled sample nucleic acids are hybridized to the probes on the microarray chips; each well of the microarray is scanned for the fluorescence intensity of each probe, the intensity of which is proportional to the expression level of that gene in the sample. The large number of microarray probes available in several commercial platforms allows for highly multiplexed assays, enabling detection of a broad range of organisms and discrimination of multiple genetic elements within the targeted species.

13.4.1.5 Stable Isotope Probing

Stable isotope probing (SIP) allows microbial identity to be linked to functional activity through the use of substrates labelled with stable isotopes. It has been used to its best advantage by labelling substrates that are used almost exclusively by the population of interest.

13.4.1.6 Metagenomics

It is the study of all the biological molecules isolated from an environmental sample based on the analysis of RNA transcripts. Study of proteins extracted from the environmental sample is known as metaproteomics, whereas metabolomics is the study of metabolites, including sugars, lipids, amino acids, and nucleotides. In the nitrogen cycle, genes encoding important reactions were detected using microarrays from soil samples and provided information on the composition and activity of the complex soil microbial community.

13.4.2 Interaction of Root Exudates with Rhizospheric Microbes

The rhizosphere is one of the most complex ecosystems on earth and is inhabited by various living organisms including nematodes, fungi, bacteria, and arthropod. Plant roots release a wide range of compounds that are involved in attracting beneficial organisms and forming mutualistic associations in the rhizosphere. The rhizosphere is the narrow region of soil that is directly influenced by root secretions and associated with soil microorganisms. Rhizosphere includes plant roots and the surrounding area of soil influenced by the roots; plants exude chemicals to effectively communicate with their neighboring soil living biota. The compounds released by roots include amino acids, polysaccharides, fatty acids, aromatic acids, aliphatic acids, enzymes, sterols, sugars, phenolics, proteins, plant growth regulators, and secondary metabolites. The most important rhizosphere mutualisms described are between plants and mycorrhizae or rhizobacteria. The most studied plant-microbe symbiotic interaction includes *Rhizobium leguminosarum* with *Pisum sativum* and *Vicia cracca* (Karunakaran et al. 2009) and *Bradyrhizobium japonicum* with *Glycine max* as host plant (Delmotte et al. 2010). Proteomic techniques like microarray and LCMS/MS and LTQ-Orbitrap MS led to evaluation of proteins involved in translation, posttranscriptional regulation, nitrogenase complex, and genes expressed related to tricarboxylic acid cycle, succinate, and pyruvate/inositol catabolism (Table 13.2).

13.5 Mycorrhizal Associations

Mycorrhizal associations are present in almost all land plants and are essential biological constituents of the rhizosphere. Mycorrhizae are grouped into two categories: endomycorrhizae and ectomycorrhizae. Arbuscular mycorrhizal fungi (AMF) are found in association with the roots of around 80% of the terrestrial plants. In this type of mycorrhizal association, root tip is affected, and germinating spores form hyphae and produce hyphopodium in root epidermis of the host plant. The establishment of AM symbioses begins with the colonization of a compatible root by hyphae produced by AM fungal soil propagules, asexual spores, or mycorrhizal roots. This intraradical colonization continues intercellularly and intracellularly, and it reaches the inner circle of cortical cells, and it networks into fungal tree branches called arbuscules (Bonfante and Genre 2010). Before colonization, it is assumed that a continuous dialogue of signals is exchanged between the symbionts to establish colonization. Since this symbiosis lacks host specificity, it has been suggested that either the plant-derived signals are conserved throughout the plant kingdom or that a broad range of related compounds are involved. Plant-released compounds like sugars and amino acids are potential fungal stimuli, but phenolic compounds, particularly flavonoids, are known as key signaling components in many plant-microbe interactions (Steinkellner et al. 2007). The AM fungi generate biologically active molecules that further mediate various signaling pathways. In the life cycle of mycorrhizal fungi, hyphal branching is a critical step as branch-inducing factor triggers morphogenesis of hyphae to ensure host root contact for the

establishment of symbiosis (De Carvalho-Niebel et al. 2002). In *Lotus japonicus*, root exudates are involved in symbiotic cross talk. The sesquiterpenes in dormant mycorrhizal fungi trigger hyphal branching (Akiyama et al. 2005). Flavonoid biosynthetic pathway in *M. truncatula* encodes for enzymes including chalcone synthase (CHS) and phenylalanine ammonia lyase (PAL) in arbuscules containing cells. Induction of these enzymes triggers high production of flavonoids that enhance mycorrhizal growth rather than antimicrobial phytoalexin production that inhibits fungal growth (Harrison 2005). Mycorrhizal fungi on perceiving chemical response from roots get stimulated and spread out to invade root tissues. However, this branching is limited to the cortex of root tissues which shows control of host plants in fungal proliferation (Garcia-Garrido and Ocampo 2002). Resting spore germinates into short explorative mycelium that increases the direct contact with host. Similarly, plant roots also receive fungal exudates that activate SYM pathway to trigger calcium spiking. This signal transduction activates cellular and transcriptional responses. Plant-fungi interaction takes place by attachment of hyphopodium on epidermal and cortical cells of roots that trigger assembly of pre-penetration apparatus (PPA). PPA is an aggregate of cytoplasm in these cells that helps in the development of penetration assembly. Once fungi colonize intracellularly in epidermis, it follows PPA's route to the inner cortex and allows the development along the root axis. This PPA mechanism is repeated on reaching the internal portion of cortical cells and allows branching on small scale. Ultimately, arbuscule forms an extensive network by branching and occupying a huge volume of cell. This strategy allows an efficient nutrient exchange (Bonfante and Genre 2010). Under this scenario, mycorrhiza-associated roots modulate plant defense responses that were activated on microbial invasion (Garcia-Garrido and Ocampo 2002). Moreover, other defense responses such as antioxidants, phenylpropanoid biosynthesis, and PR genes are also activated. These responses are weak, short termed, and strictly localized yet differ for each pathogen.

13.6 Plant Growth-Promoting Rhizobacteria (PGPR)

Plant roots communicate with microbes in a sophisticated manner through chemical communication within the surrounding of rhizosphere, thereby leading to biofilm formation of beneficial microbes and, in the case of plant growth-promoting rhizomicrobes/bacteria (PGPR), resulting in priming of defense or induced resistance in the host plant. Bacteria that benefit the plant are collectively termed as plant growth-promoting bacteria (PGPB). Rhizobacterium is usually referred to as plant growth-promoting rhizobacteria. Rhizobacteria are root-colonizing bacteria that form symbiotic relationship with many plants. PGPR enhance plant growth by direct and indirect means, but specific mechanisms involved have not all been well characterized. The PGPR benefit the plant by nitrogen fixation. They are an important group of microorganisms (bacteria and fungi) used as biofertilizers, e.g., rhizobacterium, *Pseudomonas*, *Azotobacter*, *Azospirillum*, etc. PGPB may either be rhizobacteria (PGPR) or colonize plant roots to become endophytes, with a number of species

moving between the two states (Compant et al. 2010). PGPR have been found to promote plant growth and help in sustainable agricultural development, protecting plants from phytopathogens. In other words, PGPR are beneficial bacteria inhabiting the plant rhizosphere that are directly or indirectly involved in promoting plant growth and biological control of plant diseases (Kloepper and Metting 1992). A mixture of several species of microorganisms is more effective in stimulating plant growth than a monoculture of bacteria or fungi. Plant growth-promoting rhizobacteria (PGPR) produce a variety of antibiotic compounds that inhibit a variety of Gram-negative and Gram-positive soil bacteria. The common PGPR genera in the rhizosphere include *Bacillus*, *Pseudomonas*, *Enterobacter*, *Acinetobacter*, *Burkholderia*, *Arthrobacter*, and *Paenibacillus* (Zhang et al. 2017).

PGPR colonize in the roots of plant by involving quorum sensing, a cell-to-cell communication mechanism through the release of signals to cognate receptors, thereby influencing gene expression in correlation to bacterial population density. It has now been determined that coordinated activity among microbial cells using diffusible chemical signals is a widespread phenomenon, called "quorum sensing" or "cell-to cell communication" (Greenberg 1997). Although the chemical signals and mechanisms of QS systems vary, the most prevalent form of QS signals used by plant-associated bacteria is acyl homoserine lactones (AHLs), which vary in the length, oxidation state, and degree of saturation of their acyl side chains to provide a degree of species specificity. At threshold concentrations, these AHLs form complexes with their cognate receptors, which bind to DNA and act to regulate expression of specific genes, effectively allowing populations of individual cells to act as a collective unit. In plant-associated bacteria, QS is often involved in establishing successful associations, whether they are symbiotic or pathogenic. To maintain the symbiotic relation with plants, rhizobacteria either secrete or emit molecules beneficial to the plant. These molecules, originating from the rhizosphere, are able to trigger specific changes or adjustments to the plant transcriptome. While phytohormones are growth and defense regulators produced by plants, PGPR are also able to produce these compounds that include auxins, cytokinins, gibberellins, and ABA, among others. Rhizobacteria also produce numerous volatile organic compounds comprising alkanes, alkenes, alcohols, ketones, terpenoids, and sulfur compounds.

13.6.1 Plant-Microbe Pathogenic Interaction

Most plants produce antimicrobial secondary metabolites, either as part of their normal program of growth and development or in response to pathogen attack, and those antimicrobial compounds protect plants from a wide range of pathogens (Morrissey and Osbourn 1999). During pathogenic plant-microbe interactions, the extracellular space between cell wall and plasma membrane acts as a first battle field between plants and pathogens. Bacteria, fungi, viruses, and oomycetes that colonize the living plant tissues are encased in this narrow region in the initial step of infection. Similarly, apoplastic region is believed to be an interface which mediates the first cross-link or cross talk between host and pathogen. The secreted

proteins and other metabolites derived from both host and pathogen interact in this apoplastic region and proven the final relationship between them. Comparatively fewer bacteria are considered to be soilborne plant pathogens; however, some well-studied exceptions include *Ralstonia solanacearum* (bacterial wilt of tomato) and *Agrobacterium tumefaciens*, the causal agent of crown gall disease. Thus, the disease has been called "crown gall." The microorganism is an aerobic Gram-negative bacterium that is widely distributed in soils where it grows on a variety of sugars and organic acids. The bacterium *Agrobacterium tumefaciens* produces tumors in a diverse group of dicotyledonous plants at the root-stem interface that is called the "crown" of the plant. The role of QS in the pathogenesis of *Erwinia carotovora* and *Agrobacterium tumefaciens* on their respective plant hosts is well characterized. It has been established that chemicals from the plant host contribute to infection by the tumor-inducing bacteria *Agrobacterium tumefaciens*. The signal-receptor pair (TraI/TraR) responsible for regulation of QS in *A. tumefaciens* occurs on the Ti plasmid, which is required for gall formation in host plants. An infection occurs when a segment of this plasmid is integrated into the nucleus of host plant cells, resulting in the production of opines that can then be utilized as a novel source of nitrogen and carbon. The presence of opines, which are only found in the plant tumor, then upregulates expression of the bacterial TraR gene. Thus, the QS system, which allows for conjugation and replication of the Ti plasmid, is only effectively activated after infection (Nester et al. 2005).

13.6.2 Genomics of Plant-Microbe Interaction

The secretions from plant roots help in the provision of nutrients and shelter to the microbial community around the roots. Huge amount of data and information are available for better understanding of plant-pathogen interaction at the molecular level, along with the different signaling pathways working for defense responses in plants. There is advancement at molecular level, but the regulation and changes to the plant metabolism during pathogen attacks have been recently emerging with more attention. With the introduction of sophisticated methods, genome-scale modeling is used mathematically to model the metabolism, and it is basically an in silico metabolic flux model which has been derived from the currently available genomic data. Genome-scale models are becoming quite a challenge to exploit and to analyze the phenotype during host-pathogen interactions. A number of specialized examples of many plant-bacterial and plant-fungus interactions have been extensively studied which are listed in (Table 13.1). With the advancement in genome sequencing and annotations, now it seems possible to study the genome-wide interaction of plants and its related specific pathogen. At the beginning of this decade, the transcriptomic tools like cDNA microarrays and SuperSAGE gene expression profiling have been developed to study signaling in *Arabidopsis thaliana*-*Pseudomonas syringae* pv. tomato and rice *Magnaporthe oryzae* interactions. Protein secretion system, encoded by the genome of *Pseudomonas* strain WCS, is involved in rhizosphere competence. There are six protein secretion systems in bacteria that are involved in microbe-microbe and host-microbe interactions. In genome of WCS strains, type II, III, V, and

VI secretion systems are responsible for the interaction. However, T1SS and T2SS are involved in secretion of AprA, phosphatase, lipase, and extracellular protease to facilitate nutrient acquisition. They are also involved in the secretion of bacteriocins and cyclic lipopeptides.

Millet and coworkers (2010) demonstrated that *rhizobacterium* WCS417 colonizes *Arabidopsis* roots and suppresses its immune response. This is mediated by the production of alkaline protease AprA. Interestingly, *P. syringae* and *P. aeruginosa* prevent flg22-triggered immunity by degrading flagellin monomers in *Arabidopsis* leaves. *Pseudomonas* strains suppress host immune responses via T3SS-mediated injection of effector proteins in a manner quite similar to that adopted by pathogenic microbes. However, nonpathogenic *Pseudomonas* have active T3SS gene clusters or T3SSs as they are associated with roots and do not cause any disease.

13.7 Types of Pesticide-Degrading Microorganism

In recent years, many microbes are isolated, cultured, and analyzed such as bacteria, fungi, actinomycetes, algae, and other microbial strains. Bacteria from water and sediments are sampled for the screening of endosulfan degradation from high agricultural activity areas. He found that the five bacteria genus *Klebsiella*, *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, and *Bacillus* could degrade endosulfan effectively. There are 319 actinomycetes from saline soils of Sangli District selected for carbofuran tolerance test, while only 7 strains of *Streptomyces alanosinicus*, *Streptoverticillium album*, *Nocardia farcinica*, *Streptomyces atratus*, *Nocardia vaccinii*, *Nocardia amarae*, and *Micromonospora chalcea* can grow and degrade pesticides very well.

13.8 Mechanism of Microbial Degradation of Pesticides

Pesticides in the soil could be degraded by different ways. Traditional methods included physical degradation, chemical degradation, and physical-chemical degradation, which basically caused secondary pollution. Soil microorganisms are mostly active in soils having high organic matter content as compared to soils having low organic matter contents. Increases in soil organic matter content decreased residual phytotoxicity of s-triazines. Enhanced microbial degradation is an increasingly important phenomenon affecting the degradation of pesticides in soil which ultimately cause failure of pesticide. Pesticide degradation by microbes generally involves a hydrolysis followed by metabolism and utilization of hydrolysis products as carbon or nutrient sources. Further, use of *Arthrobacter* in the enhanced degradation of isofenphos is reported and used in the enhanced degradation of both 2,4-D and EPTC.

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14 Alkaline Protease: A Tool to Manage Solid Waste and Its Utility in Detergent Industry

Vipul Kumar Yadav, Veer Singh, and Vishal Mishra

Abstract

Management of solid waste is an issue of contemporary interest worldwide. Nowadays most of the solid wastes are disposed on the land, and various anthropogenic sources like leather industry, poultry industry (feather), and other food processing industries generate a lot of biodegradable proteinaceous waste. Microorganisms have the ability to produce alkaline protease like bacteria, fungi, algae, plant and animal. This review suggests that various microorganisms are involved in the degradation of household and industrial waste by producing alkaline protease and degradation by this process not only solves the problem of waste management but also generates a source of animal feed as it yields proteinaceous by-product after degradation. Microbial sources of alkaline proteases are preferred over plant and animal sources since they have almost all characteristics which are prerequisite for biotechnological applications, like their high activity at alkaline pH (pH 10), thermostability and broad substrate specificity. Alkaline proteases are extracellular enzyme of metabolic process. This review mainly focuses on the utility of alkaline protease in management of solid waste and in detergent formulation. This review also focuses on the method to improve the capability of microorganism to increase the yield of alkaline protease.

Keywords

Alkaline protease · Biodegradation · Feather and leather waste · Detergent

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14.1 Introduction

The removal of industrial and wastewater sludge is a major concern. Nowadays sludge, which is generated from industrial and municipal waste, is disposed in sea or landfill or through incineration and other land applications (Karn and Kumar 2015). Recently the US Environmental Protection Agency (USEPA) (US Environmental Protection Agency 1991) has estimated that the half of the municipal land sites of sludge disposal area has been closed by the end of last decade and almost 50% of remaining landfills have been abandoned in the last decade due to the generation and disposal of industrial and municipals waste on the land sites.

Due to the massive growth in the population size and in the industrial activity in the last few decades, the excessive utilization of water and secondary chemical sludge generated during wastewater treatment is now an issue of contemporary interest. Therefore, it becomes necessary to degrade the secondary chemical sludge possibly by improving the efficiency of the effluent treatment plant (old conventional techniques based) or by applying new technologies, which can degrade sludge or use sludge as raw material-cum-substrate for the generation of biomolecules/enzyme which can degrade sludge.

Various industries like paper and pulp mills, textile industry and leather industry generate significant amount of biodegradable proteinaceous sludge. In addition to this, fibrous proteins such as nail, hair, feather from poultry industry and horn are also present in majority as a waste.

The biodegradation of animal leather as well as poultry feather waste not only cleans the environment, but also it generates food for animals, becomes a source of organic fertiliser for soil and also acts as a source of bioactive peptide.

The enzyme alkaline protease produced by certain microorganism play a major role in the degradation of these biodegradable wastes.

Proteases are the group of industrially important enzymes which hydrolyse or degrade the protein into small peptide or amino acid, and alkaline proteases are those enzymes which hydrolyse the protein at alkaline pH. Alkaline protease is produced by bacteria, fungi, yeast, plant and mammalian tissues (Ellaiah et al. 2002; Prakasham et al. 2005a, b; Flores-Fernández et al. 2018). They are widely used in laundry detergents, leather processing, protein recovery or solubilisation, meat tenderization and in the biscuit and cracker industries (Johnvesly and Naik 2001).

Microbial proteases are the most important hydrolytic enzymes and have been studied widely since the beginning of enzymology. Proteases not only play a key role in the cellular metabolic processes but also have an important role in the industrial community.

Alkaline protease enzymes have been widely used as detergent additives in the detergent industry. The detergent industry has now emerged as the single major consumer of several hydrolytic enzymes, which act at highly alkaline pH. Major use of detergent-compatible proteases is in the formulation of detergent. Detergents containing several sorts of enzymes like proteases, amylases and lipases are available in the international markets under brand names like Savinase, Alcalase, Opticlean, Purafect, Termamyl, Stainzyme, Maxamyl, Lumafast and Lipofast

(Novozymes report 2006; Kumar et al.1998). These enzymes as detergent additives perform catalytic activities on the various types of substrates. For example, proteases are effective against proteinaceous stains, amylases are effective against starchy stains, while lipases are effective against oily or fat stains. The enzymes which are used as detergent additives should work at alkaline pH and must be compatible with detergent. Detergents which contain protease produced by genus *Bacillus* are readily available in the international markets with the brand names like Tide, Ariel, Biz, etc. (do Nascimento and Martins 2006).

The cost of engineered alkaline proteases (EAP) produced by genetically modified microorganisms is already high; in addition to this, there is a need to improve some properties of EAP like thermostability, specificity and selectivity for the bright commercial future of alkaline proteases. Extracellular proteases have an important role in the hydrolysis of proteins in cell-free environments and also enable the cell to absorb and utilise hydrolytic products (Kalisz 1988). These extracellular proteases have been also used in a various industrial process for protein degradation (Kumar and Takagi 1999; Outtrup and Boyce 1990). Proteases are approximately 40% of the total enzyme sales in different industrial market sectors including detergent and food industry, leather industry, diagnostics and waste management. The dominance of proteases in the various industrial areas is expected to increase further in near future (Godfrey and West 1996). However, alkaline protease-based detergent shares the largest contribution of enzyme markets; these enzymes are stable and active in the alkaline pH range. The objective of this review is to discuss the application of alkaline protease in management of solid waste, its role in detergent industry and the general categories, properties and resources of alkaline proteases. In the sidelines, this review also focuses upon the study of successful approaches which improve the catalytic properties of alkaline protease, including the engineering for activity and thermostability of enzyme for industrial applications.

Furthermore, the applications of alkaline protease in various industries and along with the recent approaches for discovering and developing novel alkaline proteases by using new technologies have been discussed in the present work.

14.2 Applications of Alkaline Proteases in Waste Management

14.2.1 Degradation of Poultry Feather Waste by Alkaline Protease

It is estimated that the poultry industry processes about 400 million chicken every week, and on average each bird has 125 g of feather. The worldwide production of the feather is about 3000 tons per week. These chicken feathers are waste product of the poultry industry and create a worldwide serious issue of disposal of solid waste (Menandro 2010; Prasanthi et al. 2016; Dalev 1994). About 90% of bird feather is made of α and β keratin proteins, which consist of cysteine, lysine, proline and serine amino acid. These amino acids form disulphide bond by cross-linking with each

other, which results in thermal and insulating properties of keratins (Ward et al. 1995; Harrar and Woods 1963; Poole et al. 2009).

Prasanthi et al. (2016) characterised and analysed two fungal species *Trichophyton terrestre* and *Trichophyton mentagrophytes*. The authors reported that both fungal species have the ability to degrade the keratin protein by producing the keratinase enzyme (a type of alkaline protease), but *Trichophyton terrestre* was recommended by the author as it is less pathogenic compared to *Trichophyton mentagrophytes* (Prasanthi et al. 2016).

After degradation of feather, the remaining end product could be used as an animal feed supplement; in some countries, it is used in the form of feather meal as it is a heavy, greyish powder with very high protein content (Kumar and Takagi 1999; Dalev 1994; Dhar and Sreenivasulu 1984; Chandrasekaran and Dhar 1986; Gessesse et al. 2003; Papadopoulos et al. 1985, 1986, Steiner et al. 1983).

Mazotto et al. (2011) suggested that 62–75% of feather meal and 40–95% of feather be degraded by using three strains of *Bacillus* species isolated from feather industry. The strains were *B. subtilis* LFB-FIOCRUZ 1270, *B. subtilis* LFB-FIOCRUZ 1273 and *B. licheniformis* LFB-FIOCRUZ 1274, and a cost-effective feather by-product for fertiliser and feed was developed (Mazotto et al. 2011).

Vijayalakshmi et al. (2011) reported that alkaline protease (RV.B2.90) isolated from *Bacillus* RV.B2.90 was observed as a powerful agent for feather degradation, which remains active at extreme condition. It has ability to degrade the feather completely (more than 85%) in 24 h (Vijayalakshmi et al. 2011). Table 14.1 shows the alkaline protease-producing microorganism having ability to hydrolyse feather waste.

It became evident from Table 14.1 that bacterial species are more efficient in producing and hydrolysing the feather waste as compared to fungi due to its fast growth rate and ability to grow in extreme condition.

14.2.2 Leather Industry Waste, as a Source of Heavy Protein

Leather industries are related to everyone's life. Some requirements such as leather shoes, jackets and garments are catered by leather industry. Traditionally, the removal of hair and another unwanted subcutaneous layer from the skin/hide of animals are carried out by using chemicals. These chemicals produce secondary chemical sludge and cause environmental pollution. The alternative of this chemical treatment is application of protease enzymes. It has extensive applications in the leather and wool pretreatment. The removal of hair from the hide using alkaline protease is more pleasant and safer than the traditional methods which involved usage of strong chemical reagents like sodium sulphide. Application of the enzyme alkaline protease on leather after dehairing increases suppleness and improves the softness.

The role of alkaline proteases in the wool industry is to make "shrink-proof" wools. Silk industry is one of the least explored areas for the application of alkaline proteases. The description of alkaline protease in degumming application over the silk has been studied very scarcely, and only a few patents have been filed (Kanehisa 2000). Raw silk fibres are rough in texture due to presence of sericin, which covers

Table 14.1 Alkaline protease-producing microorganism involved in degradation of poultry feathers

Microorganisms	Group	Concentration	References
Termitomyces clypeatus	Fungi	149.4 u/ml	Majumder et al. (2015)
Aspergillus fumigates	Fungi	N. D.	Santos et al. (1996)
Trichoderma atroviride strain F6	Fungi	N. D.	Cao et al. (2008)
Trichophyton mentagrophytes	Fungi	N. D.	Prasanthi et al. (2016)
Trichophyton terrestre	Fungi	N. D.	Prasanthi et al. (2016)
Bacillus pumilus CBS	Bacteria	25,000 u/mg	Jaouadi et al. (2008)
Bacillus pumilus	Bacteria	14–16.7 u/mg	Son et al. (2008)
Bacillus subtilis	Bacteria	328 u/ml	Aftab et al. (2006)
Bacillus amyloliquefaciens	Bacteria	187 u/ml	Cortezi et al. (2008)
Bacillus megaterium F7–1	Bacteria	60 u/ml	Park and Son (2009)
Streptomyces sp. MS-2	Bacteria	11.2 u/mg	Mabrouk (2008)
Pseudomonas aeruginosa PD 100	Bacteria	434 u/mg	Najafi (2005)
Streptomyces sp. Ab1	Bacteria	9500 u/ml	Jaouadi et al. (2011)
Bacillus licheniformis RP1	Bacteria	87.73 u/ml	Haddar et al. (2011)
Bacillus pumilus A1	Bacteria	87.73 u/ml	Zouari et al. (2010)
Bacillus RV.B2.90	Bacteria	2002 u/ml	Vijayalakshmi et al. (2011)
Nesterenkonia sp. AL-20	Bacteria	452.8 u/ml	Gessesse et al. (2003)
Bacillus pseudofirmus AL-89	Bacteria	452.8 u/ml	Gessesse et al. (2003)

N. D. not defined in paper, *u* unit

the periphery of raw silk fibre, and it is about 25% of the total weight of raw silk. It is conventionally removed from the inner core of fibroin by using starch (Kanehisa 2000). This is an expensive process. An alternative and inexpensive method of degumming the silk prior to dyeing is the application of enzyme alkaline protease produced from *Bacillus* sp. RGR-14 over the silk (Puri 2001). It also adds value to the wool by providing silky lustre.

The application of alkaline protease in leather industry substantially reduces the application of the chemical pollutants such as sodium sulphide, lime and other solvents (Karn and Kumar 2015). It is known that most of the part of leather during leather processing becomes leather shavings, trimmings and splits. Only 20% of wet salted hides/skin are converted into commercial leather, while the remainder is lost and dump in wasteland as fat-soluble proteins and solid suspended pollutants (Alexander et al. 1991).

Traditionally, the waste product from the leather industry like shavings, trimmings and splits from the chrome tanning of the skins and hides have been disposed in landfills (Aftab et al. 2006).

Leather and leather-based industries are one of the most important economic sectors ranked second in export earning sector. The expected income from export of leather is more than US$ 1 billion per year. There are 700 tanneries producing about 37.2 million skins and 7.6 million hides each year in Pakistan (Aftab et al. 2006).

It has been reported that enzymatic degradation of this leather waste not only solved the problem of dumping of leather but also generated the potential

value-added products. Cabezaa et al. (1998) and Aftab et al. (2006) mentioned that alkaline protease produced by *Bacillus* species has potential to degrade protein into amino acid, which can be further used as a food additive (Cabezaa 1998; Son et al. 2008).

Table 14.2 shows the alkaline protease-producing microorganism which have the ability to hydrolyse the leather waste.

It became evident from Table 14.2 that bacterial species are massively involved in the degradation of leather waste as compared to fungal species. Among bacterial species, *Bacillus* species are more prominent source of alkaline protease production.

Bacillus species is the best understood model organism in the production of metabolites, and doing research as it is non-pathogenic in nature and has relatively large size has provided powerful tools required to investigate a bacterium from all possible aspects to enhance yield.

The overall mechanism of degradation of feather waste and leather waste has been explained in Fig. 14.1.

Figure 14.1 shows the process of utilisation and management of leather and feather waste. As its maximum constituent is protein, it can be easily hydrolyse by alkaline protease into small peptide and amino acid. After degradation, it can be utilised for feed formulation, organic soil fertiliser and manufacturing of bioactive agent.

Table 14.2 Alkaline protease-producing microorganism, which are used in the leather waste management

Microorganisms	Group	References
Termitomyces clypeatus	Fungi	Majumder et al. (2015)
Aspergillus tamarii	Fungi	Anandan et al. (2007)
Bacillus pumilus CBS	Bacteria	Jaouadi et al. (2008)
Bacillus pumilus	Bacteria	Huang et al. (2003)
Bacillus cereus MCM B-326	Bacteria	Zambare et al. (2007)
Bacillus subtilis S14	Bacteria	Macedo et al. (2005)
Bacillus subtilis	Bacteria	Pillai and Archana (2008)
Bacillus species	Bacteria	Giongo et al. (2007)
Vibrio sp. kr2	Bacteria	Alessandro et al. (2003)
Bacillus licheniformis	Bacteria	Ahamad and Ansari (2013)
Bacillus polymyxa	Bacteria	Ahamad and Ansari (2013)
Bacillus coagulans	Bacteria	Ahamad and Ansari (2013)
Actinomadura keratinilytica Cpt29a	Bacteria	Habbeche et al. (2014)
Brevibacillus sp. AS-S10-II	Bacteria	Rai and Mukherjee (2011)
Brevibacterium luteolum (MTCC 5982)	Bacteria	Thankaswamy et al. (2018)

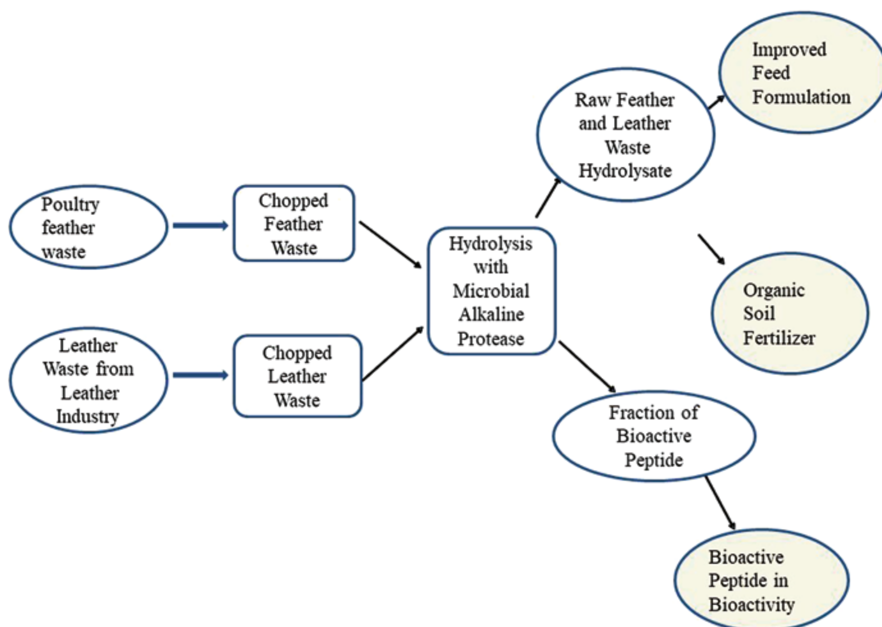

Fig. 14.1 Schematic of degradation poultry feather and leather waste by alkaline protease

14.2.3 Alkaline Protease in the Recovery of Silver from Used Photographic Film and Lith Film

The conventional method for extraction of silver from used photographic films is by burning the film; however this process of silver recovery causes a serious environmental pollution. While the silver recovery from the lith film is not possible by using this conventional process due to scarce quantity of silver in the sample, the enzymatic hydrolysis of gelatine not only helped in the recovery of silver but also enabled polyester film base for further use (Ellaiah et al. 2002; Gupta et al. 2002; Al-Abdalall and Al-Khaldi 2016; Masui et al. 2004).

It has been reported that the alkaline proteases from *Bacillus* sp. APR-4 (Kumar et al. 2002), *Bacillus* sp. B21-2 (Masui et al. 2004) and *B. coagulans* PB-77 (Gajju et al. 1996) were efficient in decomposing the gelatine layer from used photographic film and also from lith film. It has also been reported that the alkaline proteases from *Streptomyces avermectinus* NRRL B-8165 lyse gelatine layer within 15 min of its initial incubation with photographic film (Ahmed et al. 2008).

14.2.4 Alkaline Protease in Bioremediation of Environmental Pollutants

The term bioremediation refers to degradation of environmental pollutants by using the microorganism. Various enzymes which are produced by microorganisms have become a striking approach for degradation of the hazardous material from the

nature. Enzymes are safe and nontoxic bioremediation agent due to their proteinaceous nature.

Alkaline protease enzyme increases the efficiency of degradation of biodegradable substance like activated sludge. Majumder et al. (2015) used edible mushroom *Termitomyces clypeatus* for the extracellular production of alkaline protease for bioremediation (Majumder et al. 2015).

Keratinolytic protease is used as depilatory agent, which removes hair from the drains (Takami et al. 1992b). Formulation based on proteolytic enzymes produced from *Bacillus* species and thioglycolate (a disulphide-reducing agent) was principally synthesised which catalyse hair degradation which removes clogged present in pipe with hair-containing deposits, was patented, was prepared by Genex and is now also commercially available (Jacobson et al. 1985).

14.2.5 Degradation of Other Proteinaceous Waste by Alkaline Proteases

Alkaline protease helps in lowering the biological oxygen demand (BOD) in aquatic systems by solubilising the proteinaceous waste. Recently, alkaline protease opened up a new era in its applications in the management of various types of wastes originating from food-processing industries and household (Gupta et al. 2002).

Animal and human wastes are proteinaceous in nature and are present ubiquitously in environment like horn, feather, nail and hair. Certain microorganisms have the ability to convert this waste into useful biomass by forming protease enzyme, like protein concentrate or amino acids (Anwar and Saleemuddin 1998).

14.2.6 Applications of Alkaline Proteases in Detergent Industry

Alkaline proteases share a major role in the enzyme markets all over the world. Microbial alkaline proteases have numerous applications in various industrial sectors and companies worldwide, and a variety of product based on alkaline proteases have been launched in market (Lakshmi and Hemalatha 2016; Rai and Mukherjee 2009).

Alkaline proteases have large applications in the detergent industries due to its ability to remove proteinaceous stains and to carry specific benefits that cannot otherwise be gained with conventional detergent technologies (Furhan and Sharma 2014). Applications of alkaline proteases have grown up significantly, and the largest application of alkaline protease is in household laundry detergent formulations (Furhan and Sharma 2014). Alkaline protease is a proteolytic enzyme which breaks peptide bond, and that is how the proteins present in proteinaceous stain are broken into small parts of peptide or amino acid (Furhan and Sharma 2014).

Detergent based on enzyme technology is a good tool to remove the proteinaceous stain. Due to increase in demand of alkaline protease, there is a need to increase the production of alkaline protease by addition of new technology meditated by the microorganisms which can reduce the cost.

Alkaline proteases are one of the most important detergent additives used in several types of detergents ranging from those detergents which are used in household laundering to detergents used for cleaning contact lenses. The proteases share in the laundry detergents is approximately 25% part of total enzymes sale worldwide. The first enzymatic detergent was "Burnus," produced in 1913; this detergent contained crude pancreatic extract and sodium carbonate. The ideal detergent should have wide substrate specificity (which can remove protein, lipid, carbohydrate, etc. stains) towards the removal of a broad range of stains due to blood, food and other body secretions. The best performance of alkaline protease in a detergent depends on its isoelectric point (pI) value. The protease is most suitable for commercial application if its pI value match with the pH value of the detergent solution. Savinase T and Esperase alkaline protease (1996) produced by alkalophilic *Bacillus* species, these are two commercial products with very high isoelectric point (pI); and they can be used in higher pH ranges. These alkaline proteases produced from alkalophilic bacterial species are active at lower temperatures. A combination of amylase, cellulose and lipase is expected to enhance the performance of alkaline protease used in laundry detergents. Currently proteases used in detergent industries are serine proteases produced from *Bacillus* species. Alkaline proteases produced from *Conidiobolus coronatus* were found to be compatible with commercial detergents used in India (Phadatare et al.1993) and have 43% activity at 50 °C for 50 min in the presence of Ca 21 (Dhar and Sreenivasulu 1984) (25 mM) and glycine (1 M) (Bhosale et al. 1995). Table 14.3 shows the some commercial producers of alkaline proteases, manufacture company and its trade name which are used in detergent formulation.

From Table 14.3 it can be concluded that *Bacillus* species are the key players of alkaline protease production over Fungi kingdom.

Table 14.3 Commercial producers of alkaline proteases

Trade name	Make	Organism
Alcalase	Novo Nordisk, Denmark	*Bacillus licheniformis*
Savinase, Esperase	Novo Nordisk, Denmark	Alkalophilic *Bacillus* sp.
Maxacal, Maxatase	Gist-brocades, The Netherlands	Alkalophilic *Bacillus* sp.
Opticlean, Optimase	Solvay Enzymes GmbH, Germany	Alkalophilic *Bacillus* sp.
Proleather	Amano Pharmaceuticals Ltd., Japan	Alkalophilic *Bacillus* sp.
Protease P	Amano Pharmaceuticals Ltd., Japan	*Aspergillus* sp.
Maxapem	Solvay Enzymes GmbH, Germany	Protein engineered variant of alkalophilic *Bacillus* sp.
Purafect	Genencor International, Inc., USA	Genetic engineered
		Donor *B. lentus*
		Expressed in *Bacillus* sp.

14.3 Alkaline Protease-Producing Microorganisms

The distribution pattern of microorganisms is dependent on the pH value required for their optimal growth, and the majority of microorganisms proliferate almost at neutral pH values. When the pH value decreases or increases from this neutral range, the distribution of microorganisms varies. The population of alkalophilic microorganisms found in the soil is about 1/10–1/100 times of that of neutrophilic microorganism. However, some neutrophilic microorganisms show their optimum growth at extreme pH conditions. Growth under these conditions is due to the special metabolic and physiological systems, which bacterial cell has adopted from cell membrane properties and transport mechanisms which support their survival and proliferation under such adverse conditions (Krulwich and Guffanti 1983; Krulwich et al. 1990). The first obligate alkalophilic organism isolated from human and animal faeces was reported by Vedder in 1934. He described briefly about this organism and gave the name *Bacillus alcalophilus* (Vedder 1934). Nowadays, several strains of *Bacillus* species (alkalophilic) are considered for the industrial importance, mostly used as producer of alkaline proteases in laundry detergents industries (Aunstrup et al. 1972). The applications of these microorganisms in various detergent industries have prompted the isolation of alkalophilic microorganisms from a variety of artificial and natural alkaline environments (Horikoshi and Akiba 1982).

The variety of alkalophilic microorganisms, screened for industrial applications belonging to genus *Bacillus*, play predominant role in the production of alkaline proteases. *Bacillus* species which produce alkaline proteases are summarised in Table 14.4.

Table 14.4 Alkaline protease-producing *Bacillus* species

Bacillus species and their strains	References
Bacillus alcalophilus	Sharma et al. (1994)
Bacillus alcalophilus subsp. *halodurans* KP1239	Takii et al. (1990)
Bacillus circulans	Prakasham et al. (2005a, b)
Bacillus coagulans PB-77	Bryan et al. (1986)
Bacillus proteolyticus	Boyer and Byng (1996)
Bacillus subtilis var. *amylosacchariticus*	Tsuru et al. (1966)
Bacillus thuringiensis	Hotha and Banik (1997)
Bacillus sp. Ya-B	Tsai et al. (1983)
Bacillus sp. NKS-21	Takagi et al. (1992)
Bacillus pumilus	Xiubao et al. (1990)
Bacillus sp. ATCC 21536	Rahaman et al. (1988)
Bacillus amyloliquefaciens	El-Beih et al. (1991)
Bacillus amyloliquefaciens SP1	Guleria et al. (2016)
Bacillus firmus	Moon and Parulekar (1991) and Landau et al. (1992)
Bacillus intermedius	Itskovich et al. (1995)
Bacillus lentus	Bettel et al. (1992)

From Table 14.4, it can be concluded that *Bacillus* species are safer, non-pathogenic and genetically flexible. Hence they are frequently used in the detergent and other industries.

A variety of fungal species also have been reported to produce alkaline proteases (Matsubara and Feder 1971). The different types of fungal species which produce alkaline proteases enzymes have been summarised in Table 14.5. The alkaline proteases isolated from *Aspergillus* species have been studied in detail. Fungal species such as *Dendryphiella* sp. and *Scolecobasidium* sp. that produce alkaline proteases have found application in detergent industries (Pedersen et al. 1992). Yeasts (*Saccharomyces cerevisiae*) also produce alkaline proteases (e.g. *Candida lipolytica)* (Tobe et al. 1976). However, very few studies are on the alkaline protease-producing actinomycetes (Mikami et al. 1986). Table 14.5 shows the fungal species which produce alkaline protease.

It became evident from Table 14.5 that *Aspergillus* species are more efficient in producing alkaline protease as compared to other fungal species.

A. niger fermentation is "generally recognized as safe" (GRAS) by the US Food and Drug Administration under the Federal Food, Drug, and Cosmetic Act.

14.4 Production of Alkaline Proteases

Mostly alkaline proteases are produced from alkalophilic microorganisms. Essentially enzyme production can be increased by the providing optimum growth conditions to these microorganisms. The culture conditions that promote cell growth have been found significantly different from the culture conditions promoting protease production (Moon and Parulekar 1991). At industrial level, the technical media were used for the production of alkaline proteases which contained very high concentrations of complex proteins, carbohydrates and other media components

Table 14.5 Alkaline protease-producing fungal species

Fungal species	References
Aspergillus candidus	Nasuno and Ohara (1971)
Aspergillus flavus	Malathi and Chakraborty (1991)
Aspergillus fumigates	Monod et al. (1991) and Larcher et al. (1992)
Aspergillus niger	Barthomeuf et al. (1992)
Aspergillus oryzae	Nakadai et al. (1973)
Aspergillus sulphureus	Danno (1970)
Aspergillus sojae	Hayashi et al. (1967)
Aspergillus melleus	Luisetti et al. (1991)
Aspergillus sydowii	Danno and Yoshimura (1967)
Chrysosporium keratinophilum	Dozie et al. (1994)
Paecilomyces lilacinus	Belder et al. (1994)
Penicillium liliacinum no. 2093	Arai and Murao (1977)
Rhizopus oryzae	Banerjee and Bhattacharyya (1992)
Scedosporium apiospermum	Larcher et al. (1996)

(Aunstrup 1980). The economically feasible technology and research efforts are mainly focused on (i) improvement in the yields of alkaline proteases and (ii) optimization of the fermentation medium and production conditions.

14.4.1 Yield Enhancement in Alkaline Protease Production

Strain improvement of microorganism plays a key role in the production of alkaline proteases at industrial level. Wild-type strains of microorganism generally produce limited quantities of the desired alkaline protease enzyme which are useful for industrial applications (Glazer and Nikaido 1995). The alkaline protease production can be further improved by the use of antibiotics or mutagens and by using special techniques. Asporogenous mutant (not producing spore) strains of *Bacillus* species have been used for commercial production of alkaline proteases. It was observed that yield of extracellular alkaline protease production for longer duration increases fivefold by using these asporogenic mutants of *Bacillus* species (Zamost et al. 1990).

Further, the protein engineering method can improve the yield of alkaline proteases and/or subtilisins (a type of protease produce by *Bacillus* species) outside its current limitations. Nowadays, two different approaches are used for the generation of protein-engineered variants, which are site-directed and random mutagenesis. The random mutagenesis produced large number of variants of microorganisms, but its success basically depends upon the proper availability of effective screening procedures to identify the mutants with improved properties. Site-directed mutagenesis depends on the structural and biochemical data (generated by various experiment using variants) to decrease the number of variants to be created, as every variant is purified and tested individually for improvements. For production of mutated enzymes, the combination of two methods is optimally used. Different variants of microorganisms generated and identified by random mutagenesis for the production of enzyme can further be improved by using another method that is site-directed mutagenesis; this is known as advantageous mutations.

14.4.2 Enhancement in Yield of Alkaline Proteases

14.4.2.1 Cloning and Overexpression of Alkaline Proteases

The conventional mutagenesis (UV or chemical exposure) has been used to generate mutants selectively for improving protease production in microorganisms. Recombinant DNA technology (rDNA) has been also used for the construction of microorganism with genetically modified strain using selected enzyme by isolation and cloning strategies (Gupta et al. 2002). Few recombinant microbial strains with enhanced alkaline protease activity have been shown in Table 14.6.

From Table 14.6, it can be concluded that major organisms of choice for cloning and overexpression are *Escherichia coli* and *B. subtilis*. Other techniques have been also employed for the overexpression and stability of the alkaline protease gene like

Table 14.6 Overexpression of alkaline protease gene after cloning into suitable hosts

Parent strain	Host strain for cloning and overexpression	Alkaline protease gene	Plasmid vector	Enhancement in production of protease activity (x- fold)	References
Bacillus stearothermophilus NCIB 1027	*Escherichia coli* MC1061, JM109; *Bacillus subtilis* DB104	Subtilisin J	pZ124, pUC18	46	Jang et al. (1992)
Bacillus subtilis var. *amylosacchariticus*	*Bacillus subtilis* strains IS1214, 168 and 1012	Subtilisin amylosacchariticus	pHY300PLK	4–20	Yoshimoto et al. (1988)
Bacillus amyloliquefaciens	*Bacillus subtilis* I-168	Subtilisin	pBS42	200	Wells et al. (1983)
Bacillus strain YaB	*Escherichia coli* MC1061, JM109; pHY300 *Bacillus subtilis* DB104	Subtilisin (alkaline elastase YaB)	pUC18, PLK	17	Kaneko et al. (1989)
Bacillus subtilis I-168	*Escherichia coli* and *Bacillus subtilis* strains	Subtilisin E	pBS42	5	Stahl and Ferrari (1984)
Bacillus alcalophilus PB92	*Bacillus subtilis* 1-A40	Alkaline serine protease	pUB110	1.5	Van der Laan et al. (1991)
Bacillus subtilis IFO3013	*Escherichia coli*	Intracellular protease	pUB110	N.D.	Koide et al. (1986)
Bacillus sp. KSM-K16	*Bacillus subtilis* SW1214	Alkaline protease	pHY200PLK	N.D.	Hakamada et al. (1994)
Bacillus sp. G-825-6	*E. coli HB*101	Subtilisin sendai gene aprS	pUC118, pUC119	N.D.	Yamagata et al. (1995b)
Bacillus licheniformis NCIB 681	*E. coli* strains HB101, JM101; *B. subtilis 16*	Subtilisin	pBR322, pUC18,	N.D.	Jacobs et al. (1985)

N.D. not defined

site-directed mutagenesis and random mutagenesis and protein engineering, improving stability and catalytic behaviour and directed evolution of enzymes.

14.4.2.2 Molecular Approach for High-Level Expression of Alkaline Protease

Figure 14.2 shows the optimization of alkaline protease in bacterial cell system.

Construction of a plasmid for expression requires several essential regulatory elements like promoter, ribosome-binding site (RBS), transcription terminator and copy number; configuration of this element is crucial for the highest level of gene expression (Makrides 1996). In *E.coli* the promoter is situated about 10–100 bp upstream of ribosome-binding site which is controlled by regulatory gene, and it is consisted of hexanucleotide sequence. Transcription terminator is located downstream of the coding sequence, and it acts as a signal to terminate the transcription and also acts as protective element for mRNA from exonucleotide degradation (Lesnik et al. 2001). The origin of replication of a plasmid is determined as copy number (Trepod and Mott 2002). Several strategies are used to achieve high level of expression of protein which are described in Fig. 14.2.

A suitable promoter for high-level expression of gene for a particular protein must be strong and have tight regulation (Makrides 1996; Guzman et al. 1995). The promoter for large-scale production must be a chemical or thermal inducer (Chao et al. 2004; Yang et al. 2012; Wang et al. 2012).

Transcription terminator can also affect the stability of mRNA and can increase the production of protein (Newbury et al. 1987).

Codon optimization can enhance the expression of alkaline protease gene by improving the translational fidelity (Hutterer et al. 2012).

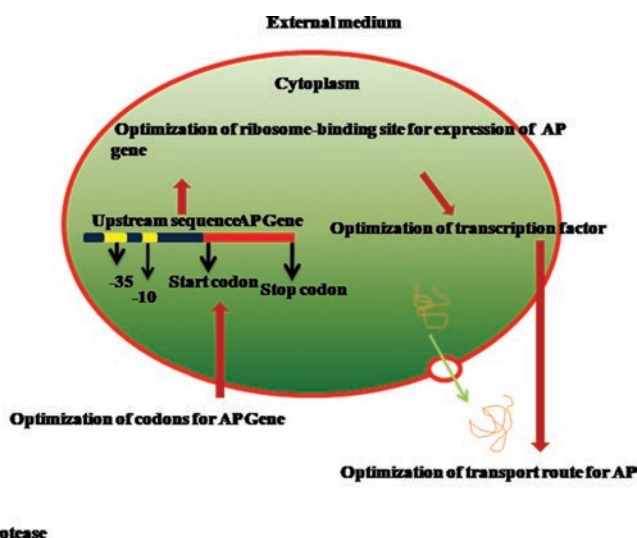

Fig. 14.2 Optimization of alkaline protease in bacterial cell system

14.5 Optimization of Fermentation Medium

Generally alkaline protease is produced by submerged fermentation, but solid-state fermentation processes are also used for the production (Chakraborty and Srinivasan 1993; Malathi and Chakraborty 1991). For commercial production of alkaline protease, the optimization of medium composition and maintenance of the balance between the different medium components and the amount of unutilised components are very crucial. Current researches mainly focus upon the estimation of the effect of various nitrogenous and carbon nutrients on the yield of alkaline protease, requirement of divalent metal ions in the fermentation medium and optimization of fermentation-cum-environmental parameters such as temperature, pH, agitation and aeration rate (Bhunia et al. 2012; Beg et al. 2002; Hameed et al. 1999; Puri 2002; Varela 1996; Lakshmi and Hemalatha 2016). However, each microorganism has its own special conditions for maximum production of the enzyme.

Table 14.7 shows the microorganisms that produce protease enzyme and their accession number.

Table 14.7 Name of enzyme and its producing organism

Enzyme	Source	Accession no.	Reference
Subtilisin E	*Bacillus subtilis*	P04189	Stahl and Ferrari (1984)
Subtilisin BPN	*Bacillus amyloliquefaciens*	Q44684	Vasantha et al. (1984)
Carlsberg	*Bacillus licheniformis*	P00780	Jacobs et al. (1985)
Subtilisin DY	*Bacillus licheniformis*	P00781	Eschenburg et al. (1988)
Alkaline protease no.221	*Bacillus clausii* strain no.221	P41362	Takami et al. (1992a, b)
M-protease	*Bacillus clausii* KSM-K16	Q99405	Kobayashi et al. (1995)
Serine protease PB92	*Bacillus alcalophilus* PB92	P27693	van der Laan et al. (1992)
Alkaline elastase YaB	*Bacillus* sp. strain YaB	P20724	Tsai et al. (1986)
Subtilisin ALP-1	*Bacillus* sp. strain NKS-21	Q45523	Yamagata et al. (1995a, b)
Serine protease Isp-1	*Bacillus subtilis* IFO3013	P08750	
Serine protease	*Bacillus polymyxa* 72	P29139	Takekawa et al. (1991)
Serine protease Isp-Q	*Bacillus* sp. strain NKS-21	Q45621	Yamagata and Ichishima (1995)
Alkaline serine protease	*Bacillus megaterium* WSH-002	AEN92144	Liu et al. (2011)
Lipases	*Micrococcus* sp. HL-2003	AAQ88181	
Alkaline protease	*Bacillus licheniformis*	AEZ67460	
Alpha amylase	*Bacillus licheniformis*	CAA01355	
Lipase	*Thermomyces lanuginosus*	O59952	Holmquist et al. (1994)
Cysteine protease	*Calotropis gigantea*	CA92037	
Clostripain	*Clostridium perfringens*	KXA14784	
Streptopain	*Streptococcus* sp.	AMH03134	
Staphylocoagulase	*Staphylococcus aureus*	BAG50050	Sakai et al. (2008)
Alkaline cellulose	*Bacillus* sp.	BAA00045	Fukumori et al. (1986)
Xylanase A	*Bacillus* sp.	BAA00055	Hamamoto et al. (1987)
Phenol hydroxylase	*Pseudomonas mendocina*	AAW66694	Heinaru et al. (2000)

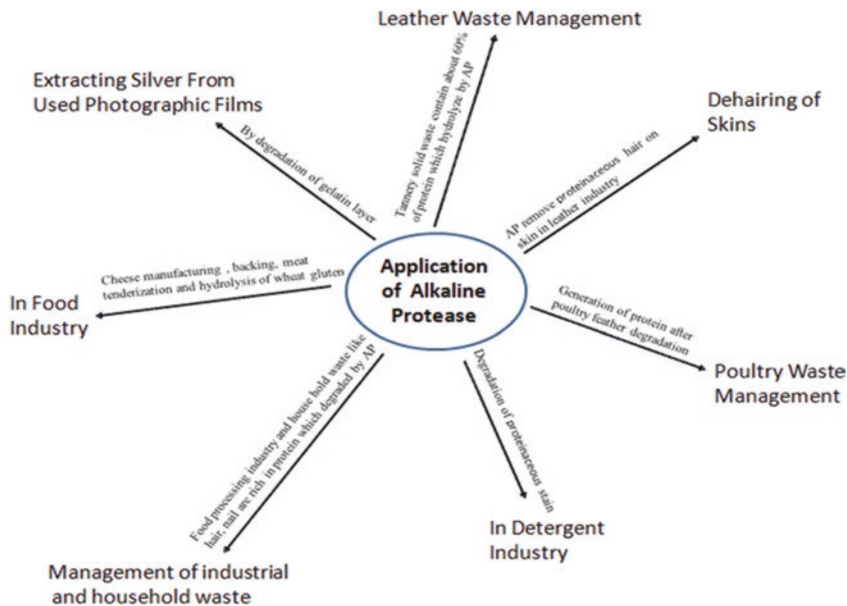

Fig. 14.3 Applications of alkaline protease in various industrial sectors

It can be concluded from Table 14.7 that *Bacillus* species are preferred by producer of alkaline protease over other bacterial species.

Figure 14.3 shows the large application of alkaline protease in leather and poultry feather waste management, silver extraction from photographic film and also in detergent industries.

14.6 Conclusion

This review focuses on the application of alkaline protease in solid waste management such as management of waste from leather industry and poultry feather waste from poultry industry. The by-product formed after degradation of leather and feather waste by alkaline protease can be used as a food for animal, as a biofertiliser for soil and as bioactive agent. This review also covers the details of microorganisms specially *Bacillus* species which are involved in production of alkaline protease enzyme. The molecular mechanism at the genetic level responsible for the high-level expression of alkaline protease from bacterial cell system is mainly regulated by promoter and codon sequence of alkaline protease gene. Alkaline protease is a good tool to clean environment. Thus tremendous prospects and role of alkaline proteases have been given importance in development of technique for solid waste management and recovery of proteinaceous food from this waste. Alkaline protease

is proteinaceous in nature; hence it is regarded as safe in handling, and it is nontoxic for humans.

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Conflict of Interest The authors declare that they have no conflict of interest.

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Heavy Metal Toxicity and Possible Functional Aspects of Microbial Diversity in Heavy Metal-Contaminated Sites

15

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Abstract

Heavy metals have emerged out as imperious category of pollutants, showing inimical effects on both human physiology and the dynamism of terrestrial and aquatic life forms and ecosystems. Depending on their oxidation states, heavy metals can be highly reactive and, therefore, toxic to the simplest to most complex organisms. Different human-based industries including metallurgical, chrome tanning, textiles, electronic, electroplating, metal culminating, fertilizer manufacture, mining, and steel and automobile industries are persuasive sources of toxic heavy metals including cadmium (Cd), gold (Au), silver (Ag), copper (Cu), lead (Pb), chromium (Cr), mercury (Hg), uranium (U), selenium (Se), zinc (Zn), arsenic (As), and nickel (Ni). All life forms including fungi, bacteria, yeasts, plants, and animals may be affected due to toxic levels of heavy metals; however, the diversity and magnitude of toxicities may vary for different organisms. Co-occurrence of different heavy metals in an ecological community may be prime mover of significant nocuous effects of the biomass/activity and diversity of soil microbiota than those instigated by single metals at high concentrations. Remediation of heavy metal-contaminated soils is getting substantial momentum and is a perplexing task as metals cannot be degraded and the

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jeopardies they stance are intensified by their moxie in the environment. Microorganisms are the first entity that endures direct and indirect impacts of hazardous heavy metals. Biochemical and molecular rejoinder of soil microbial population to heavy metal-polluted environment establish a germane model for ecological studies to appraise the influence and dynamics of environmental characteristics. Several microbes have habituated and harbor potential tolerance to detoxify heavy metal-contaminated environments at cellular level different strategies through bioaccumulation, biosorption, biotransformation, etc. for ameliorating heavy metal-contaminated sites. Therefore, universally, several researchers are trapping novel microorganisms for the isolation of competent heavy metal-tolerant bacteria.

Keywords
Heavy metals · Toxicity · Microbial diversity · Bioremediation

15.1 Introduction

Metals comprise critical components of the biome, and their biologically accessible concentrations are reliant principally on geological and biological conversion processes (Ehrlich 2002). Heavy metals epitomize inordinate environmental attention because of their prevalent use and dissemination and concretely toxicity to human beings and noxiousness to biosphere. Nonetheless, they withal comprise some elements, vital for living organisms at optimum concentrations (Alloway 1990). Heavy metals encompass essential elements, viz., iron (Fe) and zinc, along with some toxic metals like cadmium and mercury (Pires 2010). Metals can be defined as heavy metals if they exhibit density above 5 g/cm^3 (Nies 1999; Järup 2003; Wijayawardena et al. 2016); therefore, the transition elements from vanadium (V) (excluding scandium (Sc) and titanium (Ti)) to the half-metal arsenic (As), from zirconium (Zr) (excluding yttrium (Y)) to antimony (Sb), and from lanthanum (La) to polonium (Po), the lanthanides, and the actinides can be assigned as heavy metals (Issazadeh et al. 2013). Higher concentrations of these heavy metals cause a consequential environmental and health threats when the relinquishment of metals through geological decomposition and anthropogenic practices surpasses that of natural metal cycling processes (Fig. 15.1). Metal toxicity in aqueous and terrestrial ecosystems and their threat to animal and plants are of great concern of present scenario. Bacteria play a pivotal role in biogeochemical cycling of heavy metals, and their transformational competences and strategies in are of utmost interest in restoration of contaminated sites (Han and Gu 2010). Heavy metals have multifaceted utilizations and come at a consequential environmental catastrophe. Additionally, our dependence on them disseminates to result in immensely colossal inputs into environment (Han et al. 2007). Different industrial inputs and agronomic application of fertilizers, pesticides, and metal-contaminated sewage perpetuate to enrich heavy metal intensification in the soil (Herland et al. 2000).

Fig. 15.1 Pictogram showing possible health hazards of heavy metals on human health. (http://www.grida.no/graphicslib/detail/impact-of-toxic-metals_0fbe)

15.2 Sources of Heavy Metals and Their Toxicities in the Environment

Direct and indirect usage of heavy metals by industries and agricultural sectors has led to the relinquishment and infelicitous disposal of immense amounts of heavy metals in most of the ecosystems (Ansari and Malik 2007) (Table 15.1). Industrial pollution has vastly contaminated soil and water ecosystems and led to relentless environmental threat. Disseminated ecumenical industrialization is another reason for heavy metal-based ferocious environmental menace. A wide variety of chemicals, e.g., heavy metals, agricultural chemicals, industrial solvents (including chlorinated solvents), etc., have been identified in varied natural resources, viz., air, soil, and water ecosystem (Rajkumar et al. 2010). Toxic metals (e.g., Cd, Cr, Pb, Hg, As, Cu, Zn, and Ni) are inadvertently being incorporated to soils through divergent agricultural practices such as indelible usage of urban sewage sludge and industry-based practices, e.g., different methods of waste decomposition and waste incineration, and through automobile emissions. Rhizosphere can be polluted with heavy metal derived from heterogeneous sources, viz., mining and smelting of metals, electroplating wastes, gaseous emission, fuel- and energy-based industries, chemical fertilizer industry, sewage and municipal waste and mining and pesticide wastes, incongruous management of industrial wastes, incomplete amassing of utilized batteries, leakage

Table 15.1 Natural and anthropological sources of heavy metals (Lone et al. 2008)

Heavy metals	Possible source
As	Semiconductors, petroleum refining, wood preservatives, animal feed additives, coal power plants, herbicides, volcanoes, mining and smelting
Cu	Electroplating industry, smelting and refining, mining, biosolids
Cd	Geogenic sources, anthropogenic activities metal smelting and refining, fossil fuel burning, application of phosphate fertilizers, sewage sludge
Cr	Electroplating industry, sludge, solid waste, tanneries
Pb	Mining and smelting of metalliferous ores, burning of leaded gasoline, municipal sewage, industrial wastes enriched in Pb, paints
Hg	Volcano eruptions, forest fire, emissions from industries producing caustic soda, coal, peat, and wood burning
Se	Coal mining, oil refining, combustion of fossil fuels, glass manufacturing industry, chemical synthesis (e.g., varnish, pigment formulation)
Ni	Volcanic eruptions, landfill, forest fire, bubble bursting and gas exchange in ocean, weathering of soils and geological materials
Zn	Electroplating industry, smelting and refining, mining, biosolids

of landfill leachate, fortuitous spills, highway traffic, and military activities (Kim et al. 2001). Additionally, anarchic technological practices ((i) industrial, plastic, textiles, microelectronics, wood preservatives; (ii) mining, mine waste, tailings, smelting; (iii) agrochemicals, chemical fertilizers, farm yard manure, pesticides; (iv) aerosols, pyrometallurgical and automobile exhausts; (v) biosolids, sewage sludge, domestic waste; (vi) fly ash, coal combustion products) are the principal sources of heavy metal pollution in the environment along with the geogenic sources (Ma et al. 2011). These and other similar pollutants instigate accretion of metals and metalloids in rhizosphere and incite hazard to food security and public health due to soil-to-plant-to-animal/human transfer of heavy metals (Kabata-Pendias 1992; Giller et al. 1998; Del Val et al. 1999). Raucous combustion of fossil fuel, mining, and processing of mineral sources and generation of industrial effluents and treacherous discharge of sludges, biocides, and preservatives release a variety of toxic metal species into aquatic and terrestrial bionetworks, consequentially affecting flora and fauna (Gadd 1992a, 2005, 2007; Wainwright and Gadd 1997; Pokrovsky et al. 2008; Fabiani et al. 2009). Over the last decades, the annual ecumenical relinquishment of heavy metals reached 22,000 t (metric ton) for Cd, 939,000 t for Cu, 783,000 t for Pb, and 1,350,000 t for Zn (Singh et al. 2003). Anarchic industrial practices have introduced generally 100–1000-fold higher heavy metal concentrations to the environment, compared to the Earth's crust, and locally, life forms can be unleashed to even higher concentrations (Chekroun and Baghour 2013).

Heavy metal pollution along road soils is generated from motor engine and brake pad wear (e.g., Cd, Cu, and Ni) (Viklander 1998; Kannan and Ramteke 2002; Ozkutlu et al. 2009); engine emollients (e.g., Cd, Cu, and Zn) (Birch and Scollen 2003); automobile emissions (e.g., Pb) (Sutherland et al. 2003); and tire abrasion (e.g., Zn) (Smolders and Degryse 2002). Arsenic may be benign in methionine metabolism and gene silencing in animals (Hunter 2008). Arsenic and cadmium

occur naturally in minor amount in the Earth's crust and may not have been conscripted during evolutionary processes because of lower abundance equated to P and Zn, respectively, occupying adjacent elements in the respective columns of periodic table (Nawrot et al. 2006; Zhao et al. 2009). Anthropogenic activities are fundamentally responsible for the accumulation of toxic levels of As and Cd in soils (Verbruggen et al. 2009; Nascimento and Xing 2006). Metal-rich habitations withal occur due to natural confined ores and mineral deposits, and the weathering of rocks, minerals, soil, and sediments is a prodigious reservoir of metals. Recuperation of metal-contaminated habitations requires a diverse array of functional microbial community for establishment of phyto-ecology, soil reclamation, and biogeochemical cycling (Ahmad et al. 2011).

The metal toxicities may adversely affect all life forms including bacteria, fungi, plants, animals, and humans; nevertheless the degree of toxicities fluctuates for different organisms (Igwe et al. 2005) (Table 15.2). Though most of the 80 identified metals are vital to normal functional biology in humans (e.g., Fe, Mg, Zn), other metals, such as Pb, Hg, and Cd, are among the oldest toxicants to humans. Metals are unique as toxicants—they are neither produced nor ravaged by organisms, plants, or animals, because as chemical elements, they cannot be degraded beyond their elemental states (Hughes 2005). Additionally, (i) some heavy metals are paramount as micronutrients (Fe, Mo, and Mn); (ii) some toxic heavy metals are physiologically important as trace elements (Zn, Ni, Cu, V, Co, W, and Cr); and (iii) there are some heavy metals with unknown toxicities for plants and microorganisms (Hg, Ag, Cd, Pb, and U) (Schutzendubel and Polle 2002). Higher concentrations of heavy metals may pose substantial detrimental effects on ecosystems and human health due of their toxicity, accumulation in food web, and endurance in nature (Sharma et al. 2006; Tuzen et al. 2009). Some heavy metals such as Fe, Cu, and Zn are essential microelements, but others, such as Cd and Pb, have no benign physiological function and are toxic even in minute concentrations. Contamination of soil and water ecosystems by different heavy metals has consequential pertinence, as metals are not biodegradable like most organic pollutants and, therefore, accumulate in terrestrial, aquatic, and marine ecosystems (Smejkalova et al. 2003; Ortega-Larrocea et al. 2007).

15.3 Effects and Jeopardies of Heavy Metals Accumulation on Biological Systems

Metals play diverse vital roles in the physiology and metabolic activities of different life forms. Heavy metal pollution in the environmental milieu has become intense problem due to the rise in the additions of these metals (Roane and Pepper 1999). Metals are detrimental to both aquatic and terrestrial ecosystems and consequently to human health due to their mobilization and solubilization (Kabata-Pendias 1992; Del Val et al. 1999; Bailey et al. 1999; Kobya et al. 2005). Toxicity of metallic ions could be due to competition with or substituting a functional metal as well as causing conformational alteration, denaturation, and inactivation of enzymes and

Table 15.2 Physiological functions of transition metal elements, their sufficiency, toxicity, and toxicity symptoms in plants (Whites 2012)

Element	Form acquired	Physiological functions	Critical leaf concentrations (mg g^{-1} DM)		Toxicity symptoms
			Sufficiency	Toxicity	
Iron (Fe)	Fe2 + Fe3 + −chelates	Photosynthesis, mitochondrial respiration, C and N metabolism, production and scavenging of reactive oxygen species, regulation of transcription and translation, hormone biosynthesis	50–150 × 10–3	>0.5	Dark green foliage, orange-brown necrotic spots (bronzing) of older leaves, stunted growth, browning and blackening of roots
Manganese (Mn)	Mn2 + Mn-chelates	Photosystem II, enzyme activation in photosynthesis, C and N metabolism, RNA polymerase	10–20 × 10–3	0.2–5.3	Interveinal chlorosis and necrotic lesions on old leaves, blackish-brown or red necrotic spots, accumulation of black MnO2 particles in epidermal cells, drying leaf tips, stunted plants, and reduced root growth
Copper (Cu)	Cu+, Cu2 +Cu-chelates	Photosynthesis, mitochondrial respiration, C and N metabolism, protection against oxidative stress	1–5 × 10–3	15–30 × 10^{-3}	Dark green or bluish leaves followed by induced Fe chlorosis, young leaves chlorotic with dark-brown interveinal necrosis, stunted plants with short roots
Zinc (Zn)	Zn2 + Zn-chelates	Structural stability of proteins, regulation of transcription and translation, oxidoreductases, and hydrolytic enzymes	15–30 × 10–3	100–700 × 10^{-3}	Yellow leaves, chlorotic and necrotic leaf tips, interveinal leaf chlorosis, stunted plants with short roots
Nickel (Ni)	Ni2 + Ni-chelates	Constituent of urease	0.01 × 10–3	20–30 × 10^{-3}	Gray-green leaves, induced Fe-deficiency yellow or white interveinal chlorosis and necrosis in new leaves, stunted plants with short brown roots

Molybdenum (Mo)	MoO4 2–	Catalytic site of nitrate reductase, aldehyde oxidase, xanthine dehydrogenase, and sulfite oxidase	0.1–1.0 × 10–3	1	Yellowing or browning of leaves, appearance of blue purple or gold leaf pigments, reduced tillering, and root growth
Cobalt (Co)	Co2+	Nitrogen fixation	Beneficial	$10–20 \times 10^{-3}$	Pale green leaves, interveinal chlorosis in new leaves followed by induced Fe-deficiency interveinal necrosis, white leaf margins and tips, stunted plants with short brown roots and damaged root tips
Lead (Pb)	Pb2+	–	–	$10–20 \times 10^{-3}$	Dark green leaves, wilting of older leaves, stunted plants, and short blackened roots
Cadmium (Cd)	Cd2+ Cd-chelates	–	–	$5–10 \times 10^{-3}$	Brown leaf margins, reddish veins and petioles, total chlorosis, curled leaves, and brown roots. Deterioration of xylem tissues. Severe plant stunting, inhibited tillering, and reduced root growth
Mercury (Hg)		–	–	$2–5 \times 10^{-3}$	Yellow leaves, leaf chlorosis and browning of leaf points, red stems, severe stunting, and reduced root growth
Chromium (Cr)	Cr3+, (Cr2+), CrO42–	–	–	$1–2 \times 10^{-3}$	Yellow leaves, interveinal chlorosis of new leaves, necrotic spots, reduced plant height, purpling of tissues, wilting and reduced root growth

disrupting membrane integrity of cell and/or organelles (Blackwell et al. 1995). Hazardous effects of heavy metal ions have been also ascribed to their interactions with native proteins as well as from their interaction with the more swiftly accessible functional groups of proteins in nascent and other non-native form (Sharma et al. 2008). Heavy metals interrupt microbial ecology by affecting their magnification, morphology, and metabolic activities, ultimately resulting in biomass reduction and loss in diversity (Roane and Pepper 2000). Heavy metals can impair cell membranes integrity, modify enzyme-substrate specificity, disorder cellular functions, and damage DNA structure (Laws 1992; Cheng 2003; Diels et al. 2002). Toxicities of these heavy metals may occur due to replacement of crucial metals from their native binding sites or through ligand interactions (Bruins et al. 2000). Additionally, impairments in the architecture of nucleic acids, conformation of proteins, and intervention with oxidative phosphorylation and osmoregulation can be due to heavy metal toxicity (Poole and Gadd 1989; Bruins et al. 2000). Mutagenic activities of toxic heavy metals can alter DNA structure and induce carcinogenic effects in animals and humans (Knasmuller et al. 1998; Baudouin et al. 2002). Environmental and industrial exposure to heavy metals results in a stringent health hazards including prenatal and developmental defects (Sharma et al. 2008).

Exposure to high concentrations of heavy metals has been linked to deleterious effects on human health and wildlife diversity (WHO 1997). Metal-polluted soil and water can be decontaminated by chemical, physical, or biological techniques (McEldowney et al. 1993). However, chemical and physical decontamination processes irreversibly alter soil properties, exterminate biodiversity, and may relinquish the soil inadequate as a medium for plant magnification. They show a great affinity for other elements such as sulfur, disrupting enzyme functions in living cells by composing bonds with this group. Heavy metals, e.g., Cd, Pb, and Hg ions, have the capacity to bind to cell membranes, therefore disturbing cell signaling processes (Bailey et al. 1999; Manahan 2004). Heavy metals may withal trigger the generation of reactive oxygen species and free radicals, consequently leading to cellular oxidative stress (Dietz et al. 1999). Heavy metals are non-biodegradable and predispose to accumulate in the tissues of living organisms (Baird and Cann 2005; Kobya et al. 2005).

Some heavy metals are essential as they are involved in redox reactions; in electrostatic interactions to stabilize molecules, as cofactors in enzymatic reactions; and in osmoregulations (Bruins et al. 2000; Nies 1999; Hussein et al. 2005). However, some metals have no biological role and are detrimental to life forms even at very low concentration (cadmium, mercury, lead, etc.). Toxic concentrations of heavy metals in rhizosphere can adversely affect crop production, as these metals interfere with functional biology of plants, including metabolic processes, impairment of photosynthesis, and respiration and disintegration of main cell organelles, ultimately leading to plant's death (Garbisu and Alkorta 2001; Schmidt 2003; Schwartz et al. 2003). Furthermore, most of the heavy metals show low mobility in soil and are not facilely absorbed by plant roots (Garbisu and Alkorta 2001; Chen et al. 2004).

Heavy metals adversely affect bacterial viability (Pennanen et al. 1996), metabolic activity (Diaz-Ravina and Baath 1996), and population (Brookes and McGrath

1984; Fliessbach et al. 1994; Koomen et al. 1990). Microorganisms can alter reactivity and mobility of metals. Heavy metals at eminent concentrations can affect soil microbial diversity and their associated metabolic activities, subsequently influencing the soil fertility (Smith 1996). Toxic concentrations of different heavy metals greatly affect diversity, magnification, and survival of different microorganisms (Babich and Stotzky 1977). Heavy metals like other pollutants can modulate immune system function leading to immunotoxicity/immunodepression, negatively affecting an organism's health (Lawrence and McCabe 2002). For example, low doses of some heavy metals, such as Cd, Hg, and Pb, can improve immune system function, while higher doses are suppressive (Cabassi 2007; Boyd 2010).

15.4 Heavy Metals as Precarious Pollutant to Aquatic and Terrestrial Ecosystems

Heavy metals are a paramount category of hazardous pollutants (Duruibe et al. 2007) and adversely affect the health of aquatic and terrestrial ecological communities. Heavy metals are a crucial category of pollutants with both lethal and sublethal effects on living organisms, through disturbing ecological relationships and modifying chemical communication within and between species (Ramteke 1997; Sarkar and Chakraborty 2008). Accumulation of toxic heavy metals subsists both in bioavailable and non-bioavailable groups. Their mobility depends on (i) the metallic species that precipitates as cations and (ii) the other, which creates anionic component of salt (Ahemad 2012). Cd, ubiquitous and one of the most hazardous heavy metal pollutants, swiftly transported from soil to plants through vasculature, accumulates in tissues (Robards and Worsfold 1991; Bhattacharjee 1991; Christine 1997; Oliver 1997; Ortega-Larrocea et al. 2007), ultimately potentially affecting human health (Adriano 1986; Smith 1996; Jose et al. 2002; Yao et al. 2003).

Heavy metal contamination in soils is getting broadening attention not only from the public sectors but also from governmental agencies, in most developing countries (Yanez et al. 2002; Khan 2005). Soil is a dynamic system with variations in moisture, pH, and redox activities, thereby facilitating intricate composition of mineral (e.g., clay) along with organic (e.g., humic substances), aqueous, and gaseous components. Chemical and biological interactions between soil and heavy metal are based on nature of ion exchange, surface adsorption, and/or chelation reactions (Alloway 1990; Evangelou 1998). Absorption of metal and their bioavailability are reliant on soil pH (Alloway 1990).

Weathering of soil and rocks, volcanic eruptions, and wide variety of anthropogenic activities are major sources of introduction of metals into the aquatic ecosystems. These heavy metals may withal be evolved from remobilization from natural soils due to the transmutations in local redox activities and the corrosion of subsurface engineering assemblies due to perpetuated submergence under acidic groundwater (Chekroun and Baghour 2013). Soil contamination with heavy metals may withal cause transmutations in the composition of soil microbial community, adversely affecting soil characteristics (Giller et al. 1998; Kozdrój and van Elsas

2001a; Kurek and Bollag 2004; Lone et al. 2008). Different continents are severely affected with heavy metal pollution such as in Western Europe, and apparently about 300,000 sites are polluted with heavy metals (Gade 2000; McGrath et al. 2001); in the USA, 600,000 heavy metal-contaminated sites (McKeehan 2000) need reclamation; and in China approximately one-sixth of the total arable land has been contaminated with heavy metal deposition (Liu 2006). Reports also show that about 100,000 ha of croplands, 55,000 ha of grassland, and 50,000 ha of woodlands have already been lost due to heavy metal pollution (Ragnarsdottir and Hawkins 2005). However, in developing countries like India, Pakistan, and Bangladesh, only constrained data subsist for the health risk assessment (Kahlown et al. 2006; Sharma et al. 2007; Kibria et al. 2007; Khan et al. 2007; Kashif et al. 2009). Unlike organic pollutants, metals are not biodegraded, and therefore, they can persist in soil for thousands of years; nevertheless, they are transformed from one oxidation state or organic involute to another (Gisbert et al. 2003; Mahmood 2010).

15.5 Heavy Metals and Microbial Biodiversity

The estimated total number of prokaryotic species on the planet is verbalized to be 10^{30} (Dykhuizen 1998; Whitman et al. 1998). Bacteria are the most abundant and multifarious of microorganisms and constitute a paramount fraction of the entire living terrestrial biomass, ~10^{18} g (Mann 1990). Emphatically, average cellular densities of 10^{10} cells g^{-1} of soil and 10^6 cells ml^{-1} in brine have been described. Conclusively, prokaryotic biomass epitomizes more than a moiety of the total biomass on the Earth (Whitman et al. 1998). In the beginning of the 1980s, certain microorganisms were identified as hyperaccumulator of heavy metals (Vijayaraghavan and Yun 2008a, b), and some other are reported to tolerate lower heavy metal concentrations (Witter et al. 2000). Bacteria-derived biosorbent biomasses have been popularized because of the diminutive size, competence to grow under optimized environments, and their resilience to a diverse range of environmental stresses (Ansari et al. 2011). The heavy metals induced environmental stress declines diversity and metabolism of soil bacterial populations, consequently decreasing total microbial biomass, decrement in numbers of existing populations, e.g., rhizobia and a shift in microbial community structure (Sandaa et al. 1999a; Abaye et al. 2005; Wang et al. 2010). Total soil metal concentrations do not indicate a clear clue for genuine concentration in the soil solution to which soil microorganisms are exposed (EC 2003). Response/s of soil microbial population towards heavy metal contamination establishes a germane model for ecological investigation to evaluate influence of environmental physiognomies (Guo et al. 2009). Many researches have proved that metals influence microorganisms by affecting their population dynamics, morphological characters, and metabolic activities (Sandaa et al. 2001; Tsai et al. 2005; Pérez-de-Mora et al. 2006) and diversity (Dell'Amico et al. 2008). Microorganisms are the first biota that undergoes direct and indirect impacts of heavy metals, being in available forms in soil solution or adsorbed on soil colloids (Brookes 1995; Giller et al. 1998). The metals affect microorganisms

by reducing their number, biochemical activity, and diversity and transmuting the community structure (Kandeler et al. 2000; Khan and Scullion 1999; Ellis et al. 2003; Kozdrój and van Elsas 2001a).

However, metal exposure additionally leads to the establishment of tolerant microbial populations, which are often represented by several Gram-positives belonging to *Bacillus*, *Arthrobacter*, and *Corynebacterium*, as well as Gram negatives such as *Pseudomonas*, *Alcaligenes*, *Ralstonia*, and *Burkholderia* (Wuertz and Mergeay 1997; Kozdrój and van Elsas 2001b; Ellis et al. 2003). The response of the bacterial populations to heavy metal contamination depends on the concentration and bioavailability of metals itself and is dependent by multiple factors such as the type of metal and microbial species (Hassen et al. 1998). High concentrations of metals (both essential and nonessential) harm the cells by displacing the enzyme metal ions, competing with structurally cognate nonmetals in cell reactions and additionally blocking functional groups in the cell biomolecules (Hetzer et al. 2006). Microbial survival in heavy metal-polluted soils depends on intrinsic biochemical properties and physiological and/or genetic adaptation including morphological, as well as environmental, modifications of metal speciation (Abou-Shanab et al. 2007). Physical, chemical, and biological processes may coalesce under certain circumstances to concentrate metals rather than dilute them (Igwe et al. 2005). Several researchers utilizing isolation-predicated techniques have demonstrated that heavy metal contamination can cause shifts in microbial populations (Doelman et al. 1994; Roane and Kellogg 1996). The immediate toxicity of metals to soil organisms is mitigated by metal immobilization by soil colloidal components; however, heavy metals may be mobilized by local and ecumenical transmutations in soil conditions, i.e., transmutations in physical and chemical conditions of soil environment, including decrease in pH, redox potential, and enhanced decomposition of organic matter (Hattori 1996; Kelly et al. 2003). These microorganisms can withal be acclimated to intangible toxic metals from contaminated sites as they can efficiently accumulate heavy metals and radionuclides from their external environment (Ali and Wainwright 1995; Tewari et al. 2013). The soil microorganisms play a consequential role in energy flow, nutrient cycling, and organic matter turnover in terrestrial ecosystems (Bauhus and Khanna 1999). They may act as a nutrient source or sink in soils (Diaz-Raviña et al. 1993) and are involved in humification processes, degradation of pollutants, and maintenance of soil structure (Verstraete and Top 1999; Preston et al. 2001). A more perpetuated exposure to metals gradually culls resistant bacteria. On the other hand, long-term exposure to metals leads to the cull/adaptation of the microbial community which then thrives in polluted soils (Pérez-de-Mora et al. 2006; Chihching et al. 2008). Combinatorial effect of different metals may additionally show preponderant deleterious effects on soil microbial biomass/activity and diversity compared to single metals at high toxic concentrations (Renella et al. 2005; Pennanen et al. 1998). Particularly, higher accumulation of heavy metal may reduce soil microbe diversity (Gans et al. 2005; Ghosh et al. 2000), and these lesser diverse microbial communities may result low resilience to supplemental instabilities (Degens et al. 2001). However, freshly integrated heavy metals did not show any significant effect on the functional diversity of microbial

communities in humus samples of forest (Niklińska et al. 2005). Heavy metals exert noxious effects on soil microorganism (Pawlowska and Charvat 2004) and, hence, alter the diversity, population dynamics, and overall functional diversity of the soil microbiology (Smejkalova et al. 2003; Hattori 1996; Kelly et al. 2003; Nageswaran et al. 2012). Fluctuations in microbial (bacteria, algae, fungi, and yeast) respiration activities and enzymatic turnover activity may be strong markers of soil heavy metal pollutions (Szili-Kovacs et al. 1999; Broos et al. 2007), and therefore, they can serve as helpful models for assessing deleterious effects of metals at the cellular level (Avery 2001; Ramteke and Maurice 2014) (Table 15.3). Different bacterial species, isolated from same rhizosphere, polluted with heavy metals, can exhibit varied degree of tolerance (Rathnayake et al. 2009; Valls and de Lorenzo 2002). Nevertheless, microorganisms to heavy metal-polluted soil relationship is involute and contradictory (Smith 1991). However, a strategic study to evaluate dynamism of surviving indigenous populations and its diversity under artificially contaminated soil with metal salts can reveal better understanding of inherent ability of microorganisms to tolerate metals (Anyanwu et al. 2011). Heavy metal toxicity in bacteria may be accomplished through diverse variety of physical, chemical, or biological mechanisms including precipitation, complexation, adsorption, transport mechanisms, product excretion, pigments, polysaccharides, enzymes, and specific metal-binding proteins (Gadd 1992b; Maraziot 1998; Hetzer et al. 2006). Nevertheless, existence of microbial population in contaminated soils depends on inherent biochemical and functional and/or genetic adaptation including morphological changes of cells, as well as environmental reforms of metal speciation (Ehrlich 1997; Wuertz and Mergeay 1997; Bruins et al. 2000; Nies 2003). Most of the metal tolerance mechanisms may be linked to chromosomal genes and appear to be conjoined with plasmids (Cervantes and Gutierrez-Corona 1994; Wuertz and Mergeay 1997). A group of metal-chelating proteins, like metallothioneins, are very consequential in microbial metal tolerance, from metabolic perspective (Maraziot 1998; Valls and de Lorenzo 2002). Metallothioneins are small, highly conserved cysteine-rich polypeptides that are important for binding with essential metals (e.g., Zn, Cu) and non-essential metals (e.g., heavy metals) (Maraziot 1998).

Healthy and well-functioning soil ecology with efficient microbes is ergo a prerequisite for soil fertility and resilience to external factors (Brumelis et al. 2002; Hernandez et al. 2003). A functional relationship between soil microbial dynamics and plant metal

Table 15.3 Some parameters used to determine microbiological activity (Alef and Nannipieri 1995; Nannipieri et al. 2003)

Basal respiration	Dehydrogenase activity
Substrate-induced respiration	Fluorescein diacetate hydrolysis
Nitrogen mineralization	Heat output
Nitrification rate	Thymidine incorporation
Potential denitrification activity	Leucine incorporation
Nitrogen fixation	Specific enzyme activities
Adenylate energy charge	Arginine ammonification
ATP content	Dimethyl sulfoxide reduction

uptake through the time under environmental heavy metal gradients will yield more preponderant insight into the underlying processes at work (Tarah et al. 2013). In integration, microbes inhabiting in metal-contaminated soils have evolved diverse stratagems to resist themselves against metal stress (Zaidi et al. 2008). Such metal-resistant microbes can be utilized as efficient bioremediation agents (Khan et al. 2009; Ahemad 2012). Various metal-resistant bacterial species have been trapped from contaminated aquatic and terrestrial systems (Issazadeh et al. 2013). The bacterial isolates from the mine spoil and contaminated soils characterized were Gram-negatives, the group that has been often identified in metal-polluted soils. The metal-tolerant Gram-negative bacteria documented in these attempts belong to *Pseudomonas*, *Alcaligenes*, *Ralstonia*, *Burkholderia*, *Comamonas*, *Variovorax*, *Methylobacterium*, and *Flavobacterium*. In contrast, Gram-positive bacteria are known to be less tolerant to heavy metals; however, *Bacillus*, *Corynebacterium*, or other *Firmicutes* conquered some metal-contaminated soils (Seget et al. 2005).

15.6 Contemporary Tools and Techniques to Study Microbial Diversity

The understanding about prokaryotes remains incomplete and divisive in spite of advancements in the modern technologies. Owing to their copiousness and diversity, they evidently perform a significant part in many biochemical processes such as primary production, organic matter, and nutrient cycling in soil and marine environments, nitrogen fixation, and the microbial interaction with plants (Madigan et al. 2003; Doney et al. 2004) . Generally microbial diversity is recorded on the basis of the number of entities allotted to different taxa and their distribution pattern among taxa (Atlas and Bartha 1998). These tools comprised of analysis of cells at genomic and proteomic levels to acquire in vivo imaging (Table 15.4). The universal tools, in addition, are applied to gather more information regarding functional biodiversity of a microbial cells and their population with reference to the environment. In polluted sites, different microbial communities may be indulged in changing the mobility of metals via reduction, accumulation, and in situ immobilization by extracellular precipitation (Roane 1999). Several aspects are known to affect diversity, such as trophic interactions, spatial and temporal habitat heterogeneity, perturbance, and eutrophication (Torsvik et al. 2002). There are expectedly negative effects such as stress or positive effects like resource diversity or biological interactions (Fig. 15.2). Positive effects on diversity may be associated to enhanced stability, resilience, resistance to stress, and even efficiency (Griffiths et al. 1997; Nannipieri et al. 2003).

Table 15.4 Molecular methods for soil microbial diversity studies

Method	Type of information and resolution	Application in soil microbial analysis
DNA reassociation rate	Total genetic diversity, theoretical "species" number. Community "genome size." Low resolution	Global analysis of the genetic potential of communities. Comparative analysis of the overall biodiversity
Mole % G + C composition	Genetic community profile, overall community composition. Low resolution	Comparative analysis of overall changes in community composition
PCR-DGGE/TGGE sequencing of individual bands	Genetic fingerprinting of communities, affiliation of predominant community members. Intermediate resolution	Comparative analysis of community structure, spatial and temporal changes in community composition
PCR-SSCP sequencing of individual bands	Genetic fingerprinting of communities, affiliation of predominant community members. Intermediate resolution	Comparative analysis of community structure, spatial and temporal changes in community composition
PCR-T-RFLP	Community composition, relative abundance of numerically dominant community members. Intermediate resolution	Comparative analysis of distribution of microbial populations, monitoring changes in community composition
PCR-ARDRA	Genetic fingerprinting of simple communities, populations, or phylogenetic groups. Discrimination at lower taxonomic (species) levels. High resolution	Comparative analysis of microbial population dynamics
		Diversity within phylogenetic or functional groups of microorganisms
PCR-RISA	Genetic fingerprinting of populations or phylogenetic groups. Simultaneously analysis of different microbial groups. Discrimination at species or group level. High resolution	Comparative analysis of microbial population dynamics
		Diversity within phylogenetic or functional groups of microorganisms
PCR of rDNA—cloning and sequencing	Phylogenetic diversity, identification of community members. High resolution	Phylogenetic diversity of community members
PCR of functional genes—cloning and sequencing	Functional diversity. High resolution	Comparative analysis of the functional potential of communities
RNA dot/slot blot hybridization	Phylogenetic identification of metabolic active community members. Intermediate resolution	Qualitative and quantitative analysis of metabolic active populations in communities. Phylogenetic information on active community members

(continued)

Table 15.4 (continued)

Method	Type of information and resolution	Application in soil microbial analysis
FISH	Detection and specific counting of metabolic active microorganisms. Intermediate resolution	Comparative analysis of community structure
		Detection and identification of active cells. Direct phylogenetic information on community members

G + C Guanine+cytosine, *PCR* polymerase chain reaction, *DGGE* denaturing gradient gel electrophoresis, *TGGE* temperature gradient gel electrophoresis, *SSCP* single-strand conformation polymorphism, *T-RFLP* terminal restriction fragment electrophoresis, *ARDRA* amplified ribosomal DNA restriction analysis, *RISA* intergenic spacer analysis, *FISH* fluorescence in situ hybridization (Lynch et al. 2011)

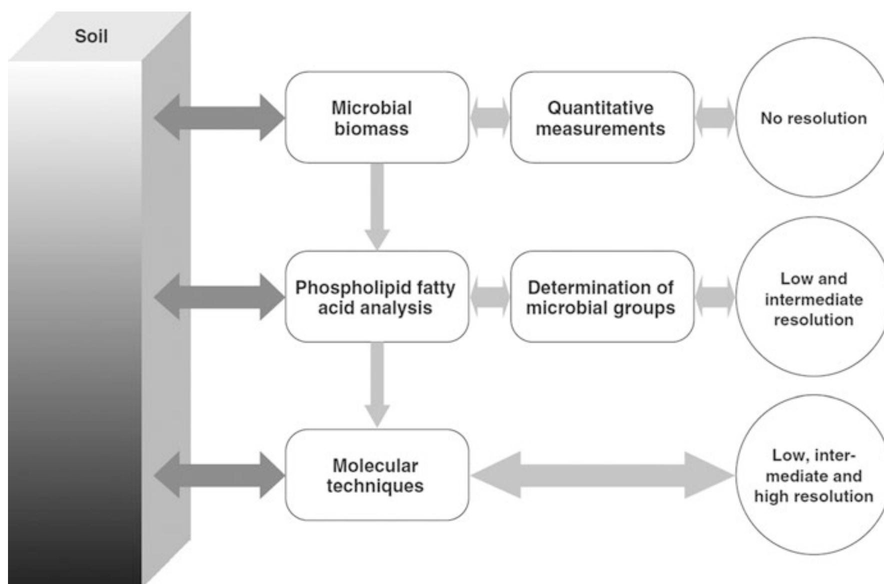

Fig. 15.2 Scientific insights provided by determining the composition of microbial communities using holistic approach in soil. (Nannipieri et al. 2003)

15.7 High-Density 16S Microarray: A Precise Technique for Close-Up Evaluation

High-density 16S microarray (PhyloChip) was used to assess indigenous microbial communities attached with metal-enriched sediments of the Coeur d'Alene River (CdAR), and clone libraries specific to bacteria (16S rRNA) were used for the analysis of ammonia oxidizers (*amoA*) and methanogens (*mcrA*). PhyloChip-based analysis gave an extensive valuation of bacterial communities and recorded the

largest number of phylotypes in *Proteobacteria* followed by *Firmicutes* and *Actinobacteria*. Additionally, clone libraries and PhyloChip presented a significant metabolic assortment in native microbial populaces by taking numerous chemolithotrophic groups, for instance, ammonia oxidizers, iron reducers and oxidizers, methanogens, and sulfate reducers in the CdAR sediments. Twenty-two phylotypes detected on PhyloChip could not be classified even at phylum level, thus suggesting the presence of novel microbial populations in the CdAR sediments. Inadequate diversity of ammonia oxidizers and methanogens in the CdAR sediments was revealed through clone libraries and also supported by the evidence that only *Methanosarcina*- and *Nitrosospira*-related phylotypes were reclaimed in *mcrA* and *amoA* clone libraries, respectively (Rastogi et al. 2011).

15.8 Thymidine Incorporation Technique

A thymidine incorporation technique was used to assess the tolerant capacity of a soil bacterial population towards Cu, Cd, Zn, Ni, and Pb. Thymidine incorporation was established as simple and fast method for checking tolerance. A direct relationship was observed between variations in community tolerance levels noted by the thymidine incorporation and plate count techniques ($r = 0.732$, $P < 0.001$). Bacterial communities isolated from metal-contaminated soil were highly tolerant in comparison with those isolated from unpolluted soil. It was not observed in case of Pb, as no description of Pb tolerance was found. An increase in the tolerance to metals other than the metal integrated to soil was additionally observed, denoting that there was multiple heavy metal tolerance at the community level. Thus, Cu pollution, in integration to improved tolerance to Cu, additionally induced tolerance to Zn, Cd, and Ni. Zn and Cd pollution enhanced community tolerance to all five metals. Ni contamination increased resistance to Ni the most, in addition, increased community tolerance to Zn, and, to some extent, improved microbial tolerance to Pb and Cd. In Pb-polluted soils, increased tolerance to other metals was recorded in the following order Ni > Cd > Zn > Cu. There was an important positive relationship between alterations in Cd, Zn, and Pb tolerance and, to some extent, between variations in Pb and Ni tolerance when all metals and changed levels were compared. The magnitude of the incrementation in heavy metal tolerance was found to be linearly associated to the logarithm of the metal concentration integrated to the soil. Threshold tolerance concentrations were assessed from these linear relationships, and alterations in tolerance could be detected at levels of soil contamination homogeneous to those reported earlier to result in alterations in the phospholipid adipose acid pattern (Diaz-Ravina et al. 1994).

Qualitative and quantitative changes in bacterial community structure have been found after exposure to heavy metals (Baath 1989; Doelman 1986; Duxbury 1985; Tyler et al. 1989). In general such shifts lead to the formation of a tolerant population. The results obtained in this study and those reported antecedently by

Frostegard et al. (Frostegard et al. 1993) seem to strengthen this fact as (i) an increment in the tolerance of the bacterial community and changes in the phospholipid fatty acid (PLFA) pattern were recorded at homogeneous metal concentrations and (ii) homogeneous effects of heavy metal integrations were deduced after the bacterial communities were grouped on the substructure of the results of a PLFA pattern analysis and tolerance pattern quantifications.

15.9 Analysis of Phospholipid Fatty Acid (PLFA) Patterns

Generally, the study of phospholipid adipose acid (PLFA) patterns is used to analyze the structure of soil microbial communities (Frostegard et al. 2011). PLFA patterns vary in various groups of microorganisms, and so, the analysis of PLFAs facilitates characterization of microbial community directly, deprived of an isolation step (Pennanen et al. 1996). PLFA pattern analysis is quite useful for the detection of any structural change in the soil microbial community due to the effect of metal pollution, soil acidification, heavy metal, and hydrocarbon pollution and soil management (Pennanen 2001; Rousk et al. 2009). Although, analysis of PLFA pattern may be challenging, as only limited PLFAs are specific for different groups of microorganisms, and thus the analysis cannot be acclimated to assess microbial diversity (Frostegard et al. 2011). Analysis of amplified and sequenced 16S rRNA genes has now playing a significant role in the structural and diversity study of soil microflora (Roesch et al. 2007; Lauber et al. 2009). High-throughput pyrosequencing is utilized not only for the assessment of the taxonomic diversity of soil microorganisms but additionally a more detailed analysis of soil microbial communities (Roesch et al. 2007; Lim et al. 2010; Tripathi et al. 2012). The utilization of this method may give a better visualization of the structure of soil microbial groups (Marcin et al. 2013). High concentrations of heavy metals adversely affected RESP and the Chao1 diversity index. PLFA analysis revealed that heavy metal pollution altered the structure of microbial communities. In contradiction, pyrosequencing has not shown any effect of heavy metal pollution on the structure of soil bacteria. The obtained results revealed that the utilization of soil microbial properties to study heavy metal effects may be tough due to influences of other environmental factors. In enormous studies local variability of soil properties may obscure the effect of heavy metals. The organization of soil microbial community depends up to large extent on soil pH. The consequence of soil pH on the structure of soil microbial community has been reported by many workers. As for instance, Rousk et al. (2009) documented a strong effect of pH change on the PLFA patterns in arable soils. Pennanen (2001) studied soils under boreal forests and found that an incrementation of humus pH transmuted the structure of microbial community towards more Gram-negative bacteria soil pH was the best fortune-teller of bacterial diversity in tropical soils across sundry land use types in Southeast Asia. The diversity of soil bacteria (Chao1) was negatively affected by heavy metal pollution. Likewise, Desai et al. (2009) reported reduction in diversity of soil bacterial communities in extensively Cr-polluted soils after a long period. Moffett et al. (2006) applied

amplified ribosomal DNA restriction analysis and found high Zn contents. The structure of soil bacterial communities up to great extent is governed by soil pH. Higher pH values support the abundance of *Chloroflexi*, *Gemmatimonadetes*, *Verrucomicrobia*, *Deltaproteobacteria*, and *Firmicutes*, profusely found in less acidic soils.

15.10 Biochemical Profiling of Soils Microbes

Soil quality depends on a combination of its physical, chemical, and biological properties, and thus microbial and biochemical characteristics are used as potential indicators of soil quality (Kennedy and Papendick 1995). The basis for the utilization of microbial and biochemical characteristics as soil quality signature is their central role in cycling of C and N and their tendency to change (Nannipieri et al. 1990). It is arduous to quantify both resistance and resilience in soil. Usually microbe-mediated procedures are most prone to the agitations in the soil, because of which the tendency of soil to recuperate from agitations can be assessed by detecting microbial activities (Pankhurst et al. 1997; Seybold et al. 1999). The relationship between microbial diversity and soil functioning, as well those between stability (resilience or resistance) and microbial diversity in soil, is not clear, as it is quite difficult to quantify microbial diversity. Soil functions are normally quantified by recording the rates of microbial processes, without having any knowledge about the microbial species efficaciously involved in the quantified process. The main problem in relationship between microbial diversity and soil function is to know the connection between genetic diversity and community structure and between community structure and function (O'Donnell et al. 2001). The relationship between microbial diversity and soil functions has been studied by methodologies based on the utilization of (i) same textured soils with different microbial composition; (ii) repetitive fumigations of soil with $CHCl_3$ to decline microbial diversity; (iii) precise biocides for killing particular soil microorganisms; and (iv) inoculation of sterile soil with soil microorganisms. Among these approaches, the second and third are destructive, while the fourth one is constructive (Griffiths et al. 2000).

15.11 Metagenomics Approach

Manufacturing, mining, and utilizations of synthetic products (paints, pesticides, batteries, industrial waste, etc.) can cause heavy metal contamination of urban as well as agricultural soil. Excess accumulation of heavy metal in soils is deadly to humans as well as other members of ecosystem. Extended acquaintance to high concentrations of heavy metals led to an enormous decline in species and allelic diversity and paramount loss of metabolic diversity (Christopher et al. 2010). It is

expected that metal pollution could change the genomics diversity of natural environments by more than 99.9% enlightening the significantly toxic effects of metal contamination (Gans et al. 2005). Microbes sustaining in heavy metal environment are directly cognate to heavy metal resistance genes which are fundamentally involved in bacterial adaptation to heavy metal stress. Metagenomics and culture-dependent analysis are being familiarized more and more in analyzing and sorting out microbial communities in heavy metal-contaminated niches. Many new microbial genes encoding for different metabolic pathways, e.g., carbon and nitrogen metabolisms and energy acquisition, in natural environments, were identified using metagenomics approach. Functional metagenomics approach was designated to identify metal resistance genes. For instance, in a study conducted on rhizospheric microbiota of acid mine drainage-acclimated plant, *Erica andevalensis* from Rio Tinto, Spain, 13 nickel resistance clones were screened and examined, coding novel proteins. The Ni resistance clones were further subdivided into two groups as per their nickel accumulating characteristics: those avoiding or favoring metal accumulation. Two clones encoding putative ABC transporter apparatus and a serine O-acetyltransferase were documented as representatives of each group, respectively (Mirete et al. 2007). Metagenomic study from microbial biofilm revealed distribution and diversity of metabolic pathway (e.g., nitrogen fixation, sulfur oxidation, iron oxidation) in acidophilic biofilms (growing in acid mine drainage in Richmond site at iron mountain, California, pH 0.83 and temperature 43 °C and high concentration of Fe, Cu, Zn, and As), in order to know the mechanism by which microbes abide extreme environments and to evaluate how this might impact the geochemistry of environmental study (Tyson et al. 2004). Bioinformatics analyses of the metagenome sequence data showed several exciting results.

Leptospirillum group III strain contains genes homologous to those assigned for biological nitrogen fixation. This understanding later resulted in the designing of a selective isolation strategy that led to the isolation of this organism (Allen and Banfield 2005).

- Genes involved in essential pathways (e.g., iron metabolism and nitrogen and carbon dioxide fixation) in the abovementioned chemolithoautotrophs were discovered.
- The genomic sequence data found genetic polymorphisms for many genes and proposed evidence for genetic recombination in the *Ferroplasma acidarmanus* population of this community.

The information of metagenome sequence established a firm background for clear-cut comparisons of microbial communities. In continuation, a latest proteomic analysis of this community recognized an abundant novel protein, a cytochrome, as a vital component to iron oxidation and acid mine drainage formation (Ram et al. 2005). These results have the possibility to design the strategy for the remediation of the sites contaminated by acid mine drainages.

15.12 Plant-Microbe Synergism for Cleaning Up Metalliferous Soils

In the course of the last two decades, bioremediation has appeared as a possible means to clean up the metal-polluted/contaminated environment (Table 15.5). The role of soil microbiota, mainly rhizospheric and endophytic microorganisms, in the development of phytoremediation techniques has to be elucidated in order to expedite the process and to optimize the rate of mobilization/absorption/accumulation of pollutants (Table 15.6). For the efficient phytoremediation of metal-contaminated soils, critical requisite is the bioavailability of metals to plant roots resulting in plant metal bioconcentration or bioimmobilization. In this respect, it may be probable to use salutary bacteria to alter the bioavailability of metals for amending phytoremediation of metal contaminants on massive scale in the environment (Ma et al. 2011). Many reports have been documented the role of rhizobacteria in heavy metal availability and accumulation in plants (Turan et al. 2012). Rhizospheric microbiota of willow (*Salix purpurea* L.) in metalliferous peat soils was associated with soil sulfate, but not with soil pH. The clone library of microbial community has shown comparable phylogenetic connections to those found in other heavy metal-contaminated soils and was dominated by associations within the phyla *Acidobacteria* (32%) and *Proteobacteria* (37%), and the remaining clones were affiliated with a wide array of phyla including *Actinobacteria*, *Bacteroidetes, Cyanobacteria*, *Gemmatimonadetes*, *Planctomycetes*, and *Verrucomicrobia*. Assorted microbial populations were present in both rhizosphere and bulk soils of these naturally metalliferous peat soils with community configuration highly correlated to the soil sulfate cycle all over the growing season symbolic of a sulfur-oxidizing rhizosphere microbial community. Results showed the significance of soil characterization for apprising bioremediation efforts in heavy metal-contaminated areas and the mutuality that microbial communities uniquely acclimated to concrete conditions and heavy metals (Tarah et al. 2013). A study was conducted on the ecology of free-living nitrogen-fixing microbial communities in rhizosphere and nonrhizosphere of pioneer plants growing on wastelands of copper mine tailings by the expression of nifH genes, employing polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE).

Twenty-two of 37 nifH gene sequences retrieved from DGGE gels clustered in *Proteobacteria* (α-*Proteobacteria* and β-*Proteobacteria*) and 15 nifH gene sequences in *Cyanobacteria*. Most nifH gene fragments sequenced were proximately cognate to uncultured bacteria and cyanobacteria and exhibited less than 90% nucleotide acid identity with bacteria in the database, suggesting that the nifH gene fragments detected in copper mine tailings may represent novel sequences of nitrogen-fixers. Non-rhizosphere tailings generally presented higher diversity of nitrogen-fixers than rhizosphere tailings, and the diversity of free-living nitrogen-fixers in tailing samples was mainly affected by the physicochemical properties of the wastelands and plant species, especially the transmutations of nutrient and heavy metal contents caused by the colonization of plant community (Zhan and Sun 2012). Soil- and plant-associated microbes on the other hand are able to leach and immobilize heavy metals in soils.

Table 15.5 Example of bioremediation of heavy metal by PGPR from polluted land (Yan-de et al. 2007; Zhuang et al. 2007; Turan et al. 2012)

Bacteria	Plant	Heavy metal	Condition	Role of PGPR	Reference
Azotobacter chroococcum IIKN-5	*Brassica juncea*	Lead and zinc	Pot experiments in green house	Stimulated plant growth	Wu et al. (2006)
				Protected plant from metal toxicity	
Bacillus megaterium HKP-1					
Bacillus mucilaginosus HKK-1					
Bacillus subtilis SJ-101	*Brassica juncea*	Nickel	Pot experiments in growth chamber	Facilitated Ni accumulation	Zaidi et al. (2006)
Brevundimonas sp. KR013	None	Cadmium	Culture media	Sequestered Cd directly from solution	Robinson et al. (2001)
Pseudomonas fluorescens CR3					
Pseudomonas sp. KR017					
Rhizobium leguminosarum bv trifolii NZP561					
Kluyvera ascorbata SUD165	*Indian mustard*	Nickel, lead, and zinc	Pot experiments growth in chamber	Both strains decrease some plants growth inhibition by heavy metals	Burd et al. (2000)
Mesorhizobium huakuii subsp. *Rengei B3*	*Astragalus sinicus*	Cadmium	Hydroponics	Expression of PCSAt gene increased ability of cell to bind Cd^{2+} approximately 9- to 19-folds	Sriprang et al. (2003)

(continued)

Table 15.5 (continued)

Bacteria	Plant	Heavy metal	Condition	Role of PGPR	Reference
Kluyvera ascorbata SUD165 and SUD165/26	Tomato, canola, perennial grasses (Graminaceae), and Indian mustard (*Brassica juncea* L. Czern.)	Cd, Zn, Cu, Ni, Co, Cr, and Pb	Pot and field experiments	Resistance to Cd, Zn, Cu, Ni, Co, Cr, and Pb and stimulation of root elongation of plant seedling	Burd et al. (1998, 2000), Dell'Amico et al. (2005) and Belimov et al. (2005)
Pseudomonas tolaasii RP23 and *Pseudomonas fluorescens* RS9, *Variovorax paradoxus*					
Rhodococcus sp. and *Flavobacterium* sp.					
Pseudomonas fluorescens 2-79	Wheat	Trichloroethylene (TCE)	Pot experiments in growth chamber	Degraded TCE with toluene o-monooxygenase	Yee et al. (1998)
Pseudomonas fluorescens F113	Alfalfa	Polychlorinated biphenyls (PCBs)	Pot experiments in growth chamber	More effectively metabolized PCBs with bph gene cloned	Villacieros et al. (2005)
Enterobacter cloacae	Tall fescue	Total petroleum hydrocarbons (TPHs)	Pot experiments in growth chamber	Promoted plant growth in the presence of environment contaminants such as TPHs	Huang et al. (2005)

Table 15.6 Some plant-associated microbial biochemical species and biochemical actions characterized for their potential to mobilize/immobilize metals and/or to alter the plant metal uptake

Types	Comment	Action	Examples
Biochemical species			
Siderophores	Low-molecular mass (400–1000 Daltons) compounds with high association constants for complexing iron	Form stable complexes with other metals, such as Al, Cd, Cu, Ga, In, Pb, and Zn	Pyoverdine, pyochelin, and alcaligin E
			Desferrioxamine B, desferrioxamine E, and coelichelin
			Pyoverdine
Organic acids	Organic acids are CHO-containing compounds characterized by the presence of one or more carboxyl groups with a maximum molecular weight of 300 daltons	Heavy metal solubilization and mobilization of mineral nutrients in the rhizosphere	Gluconic acid, oxalic acid and citric acid, tartaric acid, formic acid acetic acid
Biosurfactants	Biosurfactants are amphiphilic molecules consisting of a nonpolar (hydrophobic) tail and a polar/ionic (hydrophilic) head	Form complexes with heavy metals at the soil interface, desorbs metals from soil matrix, and thus increasing metal solubility and bioavailability in the soil solution	Di-rhamnolipid
			Lipopeptide
			Rhamnolipids
Polymeric substances and glycoprotein	Plant-associated microbes extracellularly synthesize polymeric substances (EPS), mucopolysaccharides, and proteins	Complexing toxic metals and in decreasing their mobility in the soils	Extracellular polymeric substances or cell wall lipopolysaccharides glomalin
Biochemical mechanisms			
Metal reduction and oxidization	Certain plant-associated microorganisms have the potential to alter the mobility of heavy metals through oxidation or reduction reactions	Enhance metal bioavailability in the soils through acidification reaction. Metal-reducing microbes immobilize metals within the rhizosphere soil. Fe-reducing bacteria and the Fe/S oxidizing bacteria together significantly increased the mobility of Cu, Cd, Hg, and Zn by 90%, and they attributed this effect to the coupled and synergistic metabolism of oxidizing and reducing microbes	Various cellular oxidizers and cellular reducers system, e.g., Fe-reducing bacteria and the Fe/S oxidizing bacteria
Biosorption	Microbial adsorption of soluble/insoluble organic/inorganic metals by a metabolism-independent, passive, or by a metabolism-dependent, active process	Phytostabilization of metal-polluted soils, metal accumulation, and translocation in plants through microbial biosorption/bioaccumulation	Chitin, extracellular slime, metallothioneins, P-rich amorphic material

Modified from Rajkumar et al. (2012)

The resistance to noxious metals among bacterial species is kenned (Trajanovska et al. 1997). For this, several bacterial species possess genes responsible for resistance to HM and have evolved a variety of mechanisms to reduce HM stress (Alonso et al. 2000; Van Houdt et al. 2009; Khan et al. 2009). Endophytic bacteria are the bacteria that reside within the living tissue of the host plants at least during a component of their lifetime without harming it (Wilson 1995). Mostly Gram-positive bacteria belong to genera *Bacillus*, *Paenibacillus*, *Leifsonia*, *Curtobacterium*, *Microbacterium*, *Micrococcus*, and *Staphylococcus* in different components of *Alyssum bertolonii* (a nickel hyperaccumulator, endemic to Central Italy serpentine soils). Only two groups of *Pseudomonas*-like bacteria were found as Gram-negative bacteria. Endophytes, such as *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*/*Chlorobi*, were abundant in the endosphere of a willow and were resistant to Zn and Cd (Kuffner et al. 2010). The phylogenetic analysis of copper-resistant endophytic isolates from *Elsholtzia splendens* demonstrated that they belonged to three phylotypes, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*, while *Bacillus* and *Acinetobacter* dominated the plant tissues (Sun et al. 2010). Interestingly, most of the endophytes studied so far have been shown to exhibit resistance to multiple HM (Lodewyckx et al. 2002). Such property of resistance to coalescence of metals at one time by endophytic bacteria designates that prokaryotes in general have evolved sundry mechanisms to circumvent metal toxicity. Albeit the resistance to certain amalgamations of HM is widespread in the natural environment, for certain cumulation of metals like Ni with Co, the resistance is infrequent. The resistance to amalgamation of Ni and Co is mediated by the cnr genes akin to those found in multiresistant bacteria *Cupriavidus metallidurans* (Liesegang et al. 1993). However, there are neither orthologs nor paralogs of kenned proteins for HM resistance in the betokened endophytes (Zaets and Kozyrovska 2012).

Plants dwelling in metal-contaminated soils port a diverse group of microorganisms (Idris et al. 2004; Zarei et al. 2008, 2010), capable of surviving at high concentration of metals, and are beneficial to both the soil and the plant. Among the microorganisms which are involved in heavy metal phytoremediation, the rhizospheric microbes are more significant as they can directly amend the phytoremediation process by altering the bioavailability of metal by changing soil pH, discharge of chelators (e.g., organic acids, siderophores), and oxidation/reduction reactions (Uroz et al. 2009; Wenzel 2009). Likewise, the mycorrhizal fungi, tolerant to high concentrations of metal, have been normally listed in hyperaccumulators growing in metal-contaminated soils indicating that these fungi have developed a heavy metal tolerance and that they may play a significant role in the phytoremediation of the site (Rajkumar et al. 2012).

Meanwhile in the plant-associated microbes, having the tendency to promote plant growth and/or to mobilize/immobilize metal, there has been increasing concern in the prospects of altering plant microbe interactions in metal-polluted soils. Microbial processes/metabolites promote plant growth and metal mobilization/immobilization *in vitro* but are incapable to confer favorable traits on their host in metal-polluted soils. Advance researches are largely required such as complete genome sequences for numerous environmentally useful microorganisms, elements

influencing the solubility and plant availability of nutrients/heavy metals, mechanism of microbial chelators-metal uptake in plants, and signaling processes between plant roots and microbes; these types of investigation will surely found helpful for exploring the mechanism of metal-microbes-plant interactions. Furthermore, numerous stimuli (nutrient deficiency (P, Fe) and exposure to toxic metals) in the rhizosphere could be additionally associated with metabolites (e.g., siderophores, organic acids) production. Therefore, characterizing the physicochemical-biological features of target-contaminated soils may play a significant role in the success of microbe-aided phytoremediation processes (Rajkumar et al. 2012).

The threats of heavy metal soil contamination to human, animal, and plant health and the high cost to eradicate and supersede polluted soil have led to the development of alternative technologies to improve the degraded land (Wenzel 2009). In several cases, activities of rhizosphere microorganisms have been identified as the important factor in metal availability to diverse plant species (Jin et al. 2006; Kuffner et al. 2008; Xin-Xian et al. 2009) and in phytoremediation of metal-polluted sites (He et al. 2010; Whiting et al. 2001). Rhizosphere microorganisms improve metal bioavailability and phytoremediation through various activities, such as bioleaching of metal sulfide minerals (Bosecker 1997; Fowler and Crundwell 1999), siderophore production (Joshi et al. 2006; Oliveira et al. 2006), and improved plant growth and metal uptake via mycorrhizal symbioses (Göhre and Paszkowski 2006). Highly contaminated soils are enriched with *Rhizobium* strains with many plasmids, dominated over other microbial population (Lakzian et al. 2002). Incubation studies in which a collection of different isolates of *R. leguminosarum* bv. viciae from the pristine *Rhizobium* “strain” types were reinoculated into soils with a series of long-term metal contamination supported the differences in metal tolerance between isolates with different plasmid profiles (Lakzian et al. 2007). In contrast, symbiotically efficacious *R. leguminosarum* bv. trifolii did not show adaptation to heavy metals, even after long tenure of 10 years or more (Chaudri et al. 2008; Broos et al. 2005a), indicating the differences in the proficiency of *Rhizobium* populations in various soils to acclimate to elevated metal concentrations, as is well known for higher plants (Al-Hiyaly et al. 1993). Mainly all the research done on toxicity to free-living rhizobia in soils has attested their relative sensitivity to heavy metal stress (Broos et al. 1993, 2005a, b; Chaudri et al. 2008; Giller et al. 2009).

15.13 Microbial Remediation in Heavy Metal-Contaminated Soils

Ecumenically heavy metal-contaminated environments represent prevalent environmental threats constituting a major hazard for ecosystems and human health with extravagant cleanup costs (Ansari and Malik 2007). Soil is an intricate blend of materials of mineral (e.g., clay) and organic (e.g., humic substances) inception and aqueous and gaseous components. It is a dynamic system with variations in moisture content, pH, and redox conditions. These properties interfere with the form and availability of metals (Alloway 1990). Soil and heavy metal interactions can be

understood on the substratum of ion exchange, surface adsorption, and/or chelation reactions. Biosorption can be defined as the faculty of biological materials to accumulate heavy metal from wastewaters through metabolically mediated or physiochemical pathways of uptake (Ramteke et al. 2010; Ansari et al. 2011).

Bacterial surface structures are of extreme consequentiality to understand their interactions with the circumventing environment, especially with metals. Bacteria are classified as Gram-negative or Gram-positive on the basis of their composition of the cell wall membrane. Cell walls in Gram-negative are a multilayered structure with an outer membrane containing lipopolysaccharide (e.g., lipopolysaccharide layer (LPS), phospholipids, and a diminutive peptidoglycan layer. Whereas cell walls in Gram-positive comprises 90% of peptidoglycan in several layers, with scintillas of teichoic acid conventionally present (Madigan et al. 2003; Guiné et al. 2007). These negatively charged structures are involved in interaction with metal ions (Guiné et al. 2007). The remediation of heavy metal-contaminated soils is of utmost importance and is a challenging task because metals cannot be degraded and the hazards they pose are aggravated by their persistence in the environment. Conventionally existing cleanup technologies are mostly too expensive to be habituated to recover sites, contaminated with heavy metal; moreover it adversely affects paramount properties of soils such as their texture and organic matter very oftenly (Rajkumar et al. 2010). These contaminated sites and sediments contain both prokaryotic and eukaryotic organisms and are competent enough to deal with pollution (Zettler et al. 2002; Baker and Banfield 2003). Microorganisms are very significant for recycling nutrients and heavy metals and impose a chronic stress upon the decomposer subsystem, and a variety of experimental systems and regimes have been investigated (Moffet et al. 2003). Some of these microorganisms have the capacity to alter the physicochemical conditions of their adjoining environment either by metal homeostasis, detoxification precipitation, solubilization, redox transformations, or metabolic exploitation (Bruneel et al. 2006; Hetzer et al. 2006; Guiné et al. 2007). However, as a result of (typically plasmid-encoded) heavy metal resistance, some bacterial population can acclimatize to the presence of heavy metals in loose soil and in the rhizosphere (Diaz-Ravina and Baath 1996; Malik and Jaiswal 2000; Kozdrój and van Elsas 2000), causing shifts in microbial community structure (Frostegard et al. 1993; Gray and Smith 2005; Diaz-Ravina and Baath 1996). Owing to the selective pressure imposed by the metal in the growth environment, microorganisms have evolved diverse mechanisms to resist the heavy metal stress. Numerous metal resistance mechanisms have been studied: exclusion by permeability barrier, intra- and extracellular sequestration, active transfer, efflux pumps, enzymatic decontamination, and decreased sensitivity of the cellular targets to metal ions (Poole and Gadd 1989; Bruins et al. 2000) (Figs. 15.3 and 15.4). Cadmium, copper, and zinc are among those heavy metals that are being relinquished to the environment (Roane and Pepper 1999). At present the tolerance of soil bacteria to heavy metals has been proposed as an indicator of the potential toxicity of heavy metals to other forms of biota (Olson and Thronton 1982; Hassen et al. 1998). Microbes have adapted different strategies for remedying metal-contaminated sites.

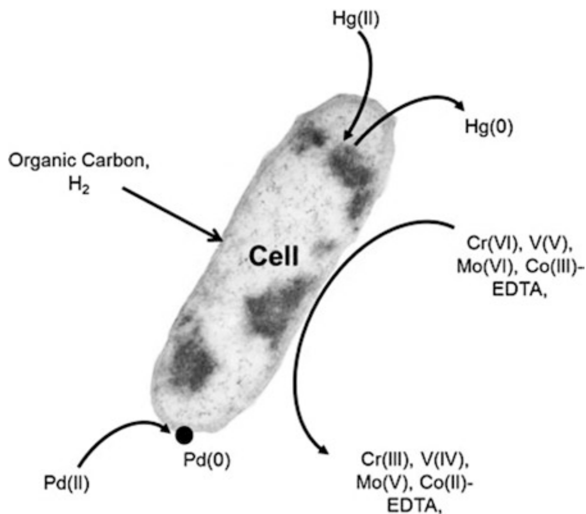

Fig. 15.3 A schematic representation of enzymatic transition metal reduction related to contaminant processes. (Watts and Lloyd 2013)

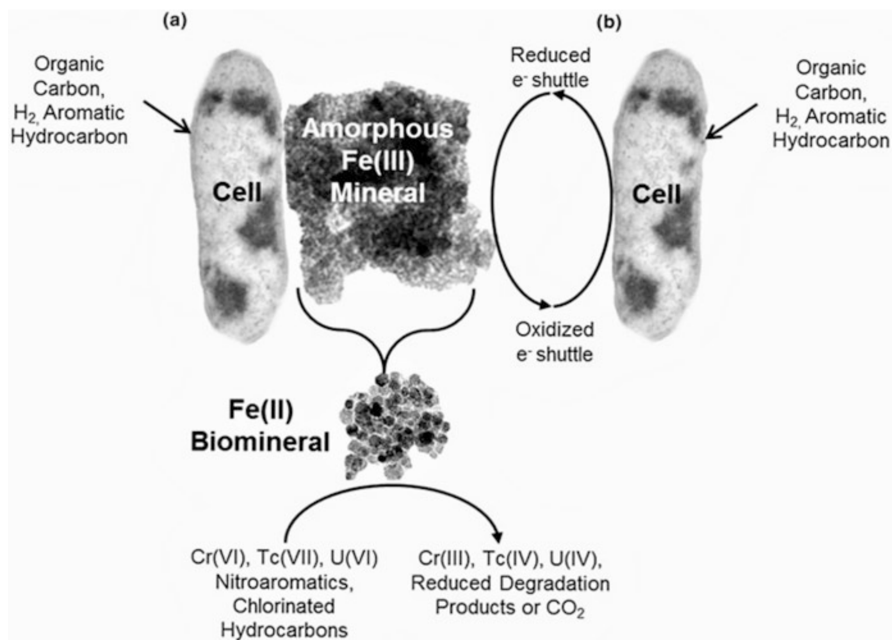

Fig. 15.4 Schematic representation of biogenic Fe(II)-mediated reduction of contaminants linked to microbial Fe(III) reduction. The two mechanisms correspond to (**a**) direct contact between the cell and Fe(III) mineral surfaces and (**b**) the utilization of an extracellular electron shuttle. (Watts and Lloyd 2013)

15.14 Biotransformation and Reduction of Heavy Metals by Microbes

The capacity of microbes to reductively transform a variety of metals has wide-reaching implicative indications for controlling the mobility of contaminants in the subsurface, resulting in the degradation of toxic organics or the reductive immobilization of metals. For instance, soluble toxic metal contaminants, including Cr(VI), Hg(II), V(V), Co(III), U(VI), Tc(VII), and Np(V), can be reduced directly and abstracted from solution by enzymatic processes, often being utilized as terminal electron acceptors during anoxic respiration (Table 15.7). In many cases these transformations can withal be mediated indirectly via reactive end products of metal reduction, including biogenic Fe(II) or Pd(0). Homogeneous indirect mechanisms for the reductive transformations of organics, such as chlorinated solvents, are withal possible, as is the enzymatic oxidation of several organic "xenobiotics," coupled directly to the reduction of metals such as Fe(III). Many of these processes occur naturally within contaminant systems, and the facility to expedite them during bioremediation applications has magnetized much recent interest. Many inorganic contaminants are redox active and can be reduced to less deleterious or less mobile forms (Lloyd 2003). Mounting research has highlighted the role of microbes in the reduction of inorganics, including transition metals and radionuclides, and often utilized as the terminal electron acceptor in anaerobic metabolism. The subsequent application of microbes with metal-reducing properties for bioremediation has led to many proposed application technologies. These typically encompass "biostimulation" strategies to enhance the activities of indigenous microbes and "bioaugmentation," via the integration of model metal-reducing microbes (Watts and Lloyd 2013).

15.15 Biotransformation and Reduction of Chromium

Cr(VI) exists mainly as $HCrO_4{}^-$, $Cr2O_7{}^{2-}$, $CrO_4{}^{2-}$, $HCr2O_7{}^-$, and $H2CrO_4$ in the environment, and Cr(VI) is more lethal than Cr(III) because of its carcinogenic nature and mutagenic effects. It is usually thought that Cr(III) is 1000 times less toxic than Cr(VI). Furthermore, Cr(VI) is highly soluble and hence mobile and naturally available in ecosystems, while Cr(III) shows a high affinity for organics, resulted in the formation of complexes that precipitate as amorphous hydroxides. Thus, the reduction of Cr(VI) to Cr(III) is a prevalent detoxification mechanism displayed by most organisms (Han and Gu 2010). The common pollutant, Cr, is associated with a substantial proportion of contaminated sites globally. The metal is redox active, consisting two valence states which predominate in environmental systems: Cr(VI) and Cr(III). Cr(III) species are predominant under acidic and moderate to reducing conditions, while Cr(VI) exists in more oxidizing and alkaline conditions (Kimbrough et al. 1999). Cr(III), however, is an essential trace element requisite for glucose and

Table 15.7 Microbial transformation of multivalence metals (Han and Gu 2010)

Metals	Possible reaction	Microorganism involved	Reference
As	Reduction of As(V) to As(III)	*Escherichia coli*, *Shewanella* sp. strain ANA-3 etc.	Stolz et al. (2006)
	Oxidation of As(III) to As(V)	*Hydrogenophaga* sp. NT-14, *Rhizobium* sp. NT-26., etc.	Stolz et al. (2006)
	Methylation of As(V) or As(III) to methylated As compound	*Desulfovibrio gigas*, *Methanobacterium formicicum*, etc.	Stolz et al. (2006)
Cr	Reduction of Cr(VI) to Cr(III)	*Pseudomonas maltophilia* O-2, *Shewanella putrefaciens* MR-1, etc.	Cheung and Gu (2003)
Fe	Reduction of Fe(III) to Fe(II)	*Geobacter metallireducens*, *Desulfuromonas acetoxidans*, *Shewanella putrefaciens*, etc.	Nealson and Saffarini (1994)
	Oxidation of Fe(II) to Fe(III)	*Leptothrix ochracea*, *Gallionella ferruginea*, etc.	Emerson (2001)
Hg	Methylation of Hg(II) to methylated compound	*Desulfovibrio desulfuricans* LS	Barkay et al. (2003)
	Reduction or oxidation demethylation of $CH_3Hg(I)$ to Hg(0) or unidentified Hg compound	*Desulfovibrio gigas*, *Escherichia coli*, etc.	Barkay et al. (2003)
	Reduction of Hg(II) to Hg(0)	*Pseudomonas aeruginosa* PU21	Barkay et al. (2003)
	Oxidation of Hg(0) to Hg(II)	*Escherichia coli*	Barkay et al. (2003)
Mn	Reduction of Mn(IV) to Mn(II)	*Geobacter metallireducens*, *Desulfuromonas acetoxidans*, *Shewanella putrefaciens*, etc.	Nealson and Saffarini (1994)
	Oxidation of Mn(II) to Mn(IV) or oxidation of Mn(II) to Mn(III) and then Mn(IV)	*Leptothrix discophora* strain SS-1, *Bacillus* sp. SG- 1, etc.	Tebo et al. (2005)
Se	Reduction of Se(VI) to Se(IV), Se(0), or even Se(-II)	*Thauera selenatis*, *Enterobacter cloacae* SLD 1a–1, *Desulfomicrobium* sp., etc.	Schroder et al. (1997) and Hockin and Gadd (2006)
	Reduction of Se(IV) to Se(0)	*Thauera selenatis*	Schroder et al. (1997)
	Oxidation of Se(0) to Se(VI) or Se(VI)	*Bacillus megaterium*	Sarathchandra and Watkinson (1981)
	Methylation of Se(VI) or Se(VI) to methylated Se(-II) compounds	*Enterobacter cloacae*, *Pseudomonas strain* Hsa.28., etc.	Ranjard et al. (2003)
U	Reduction of U(VI) to U(IV)	*Geobacter*, *Shewanella*, *Desulfovibrio*, etc.	Wall and Krumholz (2006)
	Oxidation of U(IV) to U(VI)	*Acidithiobacillus ferrooxidans*, *Thiobacillus denitrificans*	Di Spirito and Tuovinen (1982) and Beller (2005)

lipid metabolism (Wang 2000). In the meantime, the first documented isolations of organisms capable of enzymatic microbial Cr(VI) reduction in the late 1970s, *Pseudomonas dechromaticen* (Romanenko and Koren'kov 1977) and *Pseudomonas chromatophila* (Lebedeva and Lialikova 1979), a diverse group of Cr(VI)-reducing microbes have been recognized (Cervantes et al. 2007). Enzymatic Cr(VI) reduction has been established under aerobic and anaerobic conditions, with some bacteria displaying reduction under both conditions, for example, *Escherichia coli* ATCC 33456 (Shen and Wang 1993). Certainly, a proteomics study correlated Cr(VI) stress in *Pseudomonas aeruginosa* to overexpressed generation of the ROS detoxification protein glutathione (Kilic et al. 2009). *Pseudomonas stutzeri*, isolated from a foundry soil, was found to be resistant to the toxic effect of chromium up to 1 mM and reduce Cr(VI) up to 100 μM, anaerobically (Tsai et al. 2005).

15.16 Biotransformation of Arsenic

In natural environment, arsenic occurs mainly in four oxidation states: As(V), As(III), As(0), and As(-III). As(V) and As(III) are the most abundant inorganic species in nature. As(V) dominates in oxygen-enriched aerobic atmospheres, whereas As(III) predominates in reducing anaerobic environments such as groundwater. Elemental arsenic is found very rarely, and arsines have been occurred in fungal cultures and vigorously reducing environs. At neutral pH, As(V) subsists as anionic species, $H_2AsO_4^{\ -}$ and $HAsO_4^{\ 2-}$ (pKa1 = 2.24, pKa2 = 6.94, and pKa3 = 12.19), whereas As(III) is neutral (pKa1 = 9.29). Consequently, As is more mobile than arsenate in soil and sediment environments. Furthermore, As(III) is at most recent times more lethal than As(V) (Han and Gu 2010). A huge number of microbial communities have tendency to use either the oxidized or the reduced arsenic forms as electron acceptors in their different metabolic reactions. Moreover, large number of microorganisms can prevail in its toxic environment through the *ars* gene (Oremland and Stolz 2003). More likely, As-resistant organisms were isolated from arsenic-contaminated environments, but in vitro cultures of bacterial strains such as *Escherichia coli* and *Pseudomonas aeruginosa* also display resistance to high concentrations of As (Jackson et al. 2005). Phylogenetic analysis of the genes playing the role as arsenic resistance denotes that they might be more abundant in microorganisms than previously expected (Jackson and Dugas 2003; Jackson et al. 2005). This fact revealed that microorganisms in an arsenic-free environment may exhibit arsenic resistance. These microorganisms can be involved in altering arsenic into different electronic valence forms that may turn out to be more available via pollution and may be significant in local emission strategies and represent a background reservoir of As-resistant microorganisms (Jackson et al. 2005). Oxidation of As by bacteria has been detected in acid mine drainage (AMD) and in homogeneous atmospheres (e.g., sultry springs) (Battaglia-Brunet et al. 2002; Oremland and Stolze 2003; Bruneel et al. 2006).

15.17 Biotransformation and Reduction of Selenium

Selenium, an essential trace element, is incorporated into an amino acid (selenocysteine), although it may be deadly to humans at higher concentrations. Selenium subsists in the environment in various oxidation states in both inorganic and organic forms (Han and Gu 2010). Naturally the metalloid, Se, is present in soils and waters at variable concentrations, ranged from "selenium deficient" to "seleniferous" (Garbisu et al. 1996). Se occurs in various valence states Se(VI), Se (IV), and Se(0) under environmental conditions (Dungan and Frankenberger 1999). Se(0) is insoluble, but the first two species primarily form the soluble and toxic anions, Se(VI)O^{42-} and Se(IV)O^{32-} (Masscheleyn et al. 1990), thus creating reductive stabilization to Se(0) and further to Se(-II) (selenide), a desirable remediation reaction (Lenz and Lens 2009). In a study conducted on a facultative isolate from seleniferous agricultural wastewater, *E. cloacae* was identified as a potent reducer of SeO42- via the intermediary SeO32- to nanoparticulate Se(0) (Losi and Frankenberger 1997). *S. oneidensis* and *G. sulfurreducens* have been reported to have potential for reductions of Se(IV) (Pearce et al. 2009). *G. sulfurreducens* was found capable to reduce Se(IV) to Se(0) and then further to Se(-II), whereas *S. oneidensis* was identified as a reducer of Se(IV) to nanoparticulate Se(0) phases. Further findings of this study also indicated the important role of c-type cytochromes and ferredoxin in the formation of Se(0) nanoparticles. Additionally reduction of Se(0) to Se(-II) was also observed in other bacterial strains such as *Bacillus selenitireducens* (Herbel et al. 2003) and *Veillonella* (Pearce et al. 2008). The significant role of metal reduction through microbes in the bioremediation of metal-contaminated environments is now well established. The emergent "omics" and other advanced analytical techniques to environmental microbiology are progressively being used to provide better visions into bioremediation processes. Many investigations are currently at a "proof-of-concept" laboratory scale; only some renowned exceptions have been applied to solve field or industrial-scale problems. These can lead to the complete oxidation of noxious organics to the irreversible reduction of toxic metals and radionuclides (Law et al. 2010; McBeth et al. 2007; Watts and Lloyd 2013).

15.18 Mechanisms of Microbial Fe(III) Reduction

The driving between the two states, i.e., oxidized (Fe(III)) and reduced (Fe(II)), is microbially mediated reaction, in which Fe(III) acts as a terminal electron acceptor in anaerobic metabolism and Fe(II) as an electron donor in both anaerobic and aerobic processes (Weber et al. 2006). Microbial tendency to reduce Fe(III) as an electron acceptor during metabolic reactions has been already proven. Organisms having potential of Fe(III) reduction coupled to growth was recorded later with the isolation of *Geobacter metallireducens* (Lovley and Phillips 1988). This is a substantial finding as members of *Geobacteraceae* family are well known within the natural environments and are proficient in coupling Fe(III) reduction to the oxidation of acetate and various other organic substrates (Lovley et al. 2004). Initially it

was proposed that outer membrane cytochromes were a crucial component for electron transfer of *Shewanella* spp. (Myers and Myers 1992). The electron transfer apparatus, in the cellular model of Fe(III) reducers, *Geobacter* spp. and *Shewanella* spp., have now been well established (Lovley et al. 2004; Hartshorne et al. 2009). The subsequent biogenic Fe(II) not only get adsorbs to the pristine Fe(III) phase but can also form the distinct secondary biominerals (Lloyd et al. 2008). The mineralogy of the resulting Fe(II) phase is known by a complex interaction of microbial and geochemical controls (Hansel et al. 2003). The tendency of a organism to connect Fe(III) reduction to the oxidation of aromatic hydrocarbons was firstly described for the bacterium *G. metallireducens* (Lovley et al. 1989a), which was isolated from a hydrocarbon-contaminated aquifer, and was able to acquire energy from oxidation of benzoate, phenol or p-cresol, and toluene, using Fe(III) as the chief electron acceptor (Lovley and Lonergan 1990). The promotion of Fe(III) reduction exploiting electron-transporting compounds was also linked to an increased rate of aromatic oxidation within the subsurface (Borch et al. 2010). Several studies have established this through the utilization of the humic analogue AQDS (anthraquinone-2,6-disulphonic acid) (Snoeyenbos-West et al. 2000; Jahn et al. 2005). Earlier Fe(II)-mediated reduction of an enzymatically obstinate pollutant, a nitroaromatic compound, utilizing Fe(II) produced from the oxidation of BTEX (benzene, toluene, ethylbenzene, and xylene) compounds by *G. metallireducens* was reported (Tobler et al. 2007). Biogenic magnetite was also revealed to be efficient for the reduction of the routine soluble inorganic contaminant Cr(VI) to the insoluble and nontoxic Cr(III) and the reduction of the fission product Tc(VII) to insoluble Tc(IV) (Cutting et al. 2010; Watts and Lloyd 2013).

15.19 Reduction of Hg(II)

Along with the natural emission, anthropogenic emissions (2479 Mg/year^{-1} in 2006) (Streets et al. 2009) resulted in a large contribution to the Hg biogeochemical cycle, mainly the consequence of fossil fuel combustion, mining, gold and nonferrous metal generation, and the chlor-alkali processes (von Canstein et al. 2002). The toxicity of Hg is connected with its valence state, with ionic Hg(II) supposed to be the most toxic in comparison to the Hg(0) which is less toxic (Clarkson 1997). The toxicity of Hg(II) is attributed to its ability to bind to the key metabolic enzymes and thus make them inactive (Barkay et al. 2003). Natural existence of Hg and its acute toxicity resulted in the evolvement of a highly conserved bacterial Hg(II) detoxification mechanism (Wagner-Dobler et al. 2000), which comprises of uptake, followed by the intracellular reduction of toxic Hg(II) to the far less toxic Hg(0), that is subsequently ejected owing to its high vapor pressure and low solubility (Barkay et al. 2005). The mer detoxification mechanism actively conveys Hg(II) into the cell via a series of specific uptake proteins comprising periplasmic MerP (in Gram-negative bacteria) and the cytoplasmic membrane-bound proteins MerT, MerC, MerF, and Mer E (Barkay et al. 2003). When inside the cell, the Hg(II) is transferred

to the MerA enzyme via redox buffers, for example, glutathione or cysteine, or directly from the MerT membrane protein (Barkay et al. 2003). The MerA enzyme is an NAD(P)H-dependent mercuric reductase accountable for the reduction of the Hg(II) ion to Hg(0), which then expel out of the cell membrane through passive diffusion (Barkay et al. 2003; Watts and Lloyd 2013).

15.20 Reduction of V(V)

Vanadium is rather copious transition metal used primarily in the metallurgy industry (Teng et al. 2006). In environmental condition vanadium exists in three valence states, V(III), V(IV), and V(V), with a variety of ion pairs, complexes, and polymers related with each (Wanty and Goldhaber 1992). Remediation of mobile, toxic V(V) lies on the stabilization through reductive precipitation to the less mobile V(IV). *Micrococcus lactilyticusI* showed enzymatic microbial reduction resulting the reduction of vanadate (V) anion, to the vanadyl (IV) cation, with H2 as the electron donor (Woolfolk and Whiteley 1962). Reduction mechanism along with growth, through V(V) as the sole electron acceptor, has already been demonstrated in the anaerobic metal reducers *G. metallireducens* (Ortiz-Bernad et al. 2004b) and *S. oneidensis MR-1* (Carpentier et al. 2005; Carpentier et al. 2003). V(V) reduction studies in *G. metallireducens* offered indication of in situ V(V) reduction at the Rifle, Colorado, field site (Ortiz- Bernad et al. 2004b). An *Enterobacter cloacae* EVSA01, a V(V)-reducing bacterium, was isolated from a gold mine of South Africa, proposing an extensive existence of V(V) reducing bacteria (van Marwijk et al. 2009; Watts and Lloyd 2013).

15.21 Reduction of Co(III)

The radioactive isotope ^{60}Co is a major pollutant at numerous energy sites, normally, in the form of the Co(II) or Co(III) valence states, although the Co(III)-EDTA complex is found more stable in the environmental condition (Blessing et al. 2001). Reduction to Co(II)-EDTA can be a promising remediation reaction which on dissociation releases ionic Co(II) and sorbs to iron oxides (Gorby et al. 1998). Dissimilatory reduction of the Co(III)-EDTA complex coupled to growth, proposed to be an energy yielding process and was first observed in Fe (III) reducing bacterium *G. sulfurreducens* type strain PCA (Caccavo et al. 1994). Subsequently, other bacteria exhibiting Co(III)-EDTA reduction have been studied: *S. algae* (Gorby et al. 1998), *S. oneidensis* (Hau et al. 2008), and *Desulfovibrio vulgaris* (Blessing et al. 2001). The mechanism of Co(III)-EDTA reduction was investigated for S. *oneidensis* (Hau et al. 2008). This study demonstrated, utilizing mutants, that the Mtr extracellular respiratory pathway was paramount for Co(III) reduction (Watts and Lloyd 2013).

15.22 Biotransformation of Uranium (U)

Uranium is found in the oxidation states extending from U(III) to U(VI), U(VI) and U(IV) being the most stable species, subsisting in the environment. U(VI) is mainly found in the oxic surface waters, and UO_2^{2+} (uranyl) forms stable, soluble complexes with ligands such as phosphate, carbonate, and humic substances. In natural waters, the U(VI) solubility increases by several folds at higher pH values, owing to complexation with carbonate or bicarbonate. In contrast, U(IV) is predominant in the anoxic conditions and is existing primarily as an insoluble uraninite (UO_2). Thus, reduction of the soluble uranyl to the insoluble uraninite seems to be an efficacious methodology to immobilize uranium in the anoxic environment to decrement the potential relinquishment of the mobile species (Han and Gu 2010) (Fig. 15.5).

Bacteria such as *Arthrobacter*, *Bacillus*, and *Lactobacillus* sp. can accumulate immensely huge quantities of U from aqueous systems. Though, these bacterial free cells are not used again due to their mechanical variability and susceptibility to cell degradation. The tendency of the immobilized *Arthrobacter* cells to adsorb U did not decrement after six recurrences of adsorption-desorption cycles. Therefore, immobilized microbial cells seem to have excellent handling features and can be used continually in adsorption-desorption cycles. Some specific microbes have a high U accumulating capacity, which proposes its probability to be utilized for the abstraction of U from U mine tailings, U refining wastewater, and other waste areas. *Lactobacillus* and *Bacillus* sp. sequestered from Japanese U deposits abstracted 88.1% and 74.4% U, respectively. The amount of U abstracted by *Chlorella* cells from solutions containing 1.196×10^{-3} M sodium hydrogen carbonate was less at pH values above 6 than at pH 5. Albeit *Lactobacillus* sp. abstracted 36.2% of U from seawater, it abstracted proximately twice as much (70.2%) when the seawater

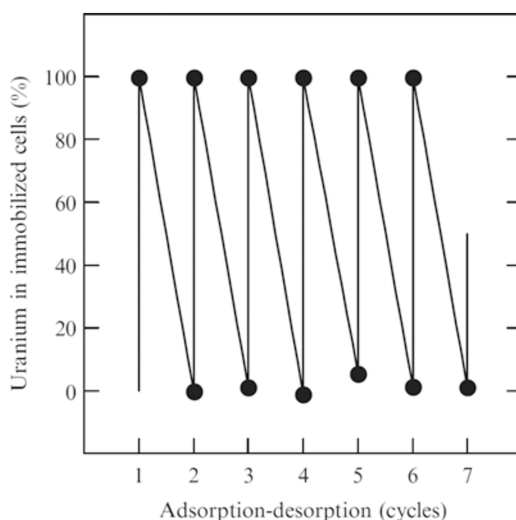

Fig. 15.5 Test of repeated uranium adsorption-desorption using immobilized *Arthrobacter*, US-10 cells. (Han and Gu 2010)

was decarbonated. *Arthrobacter* and *Bacillus* cells, which can abstract considerably huge amounts of U from nonsaline dihydrogen monoxide, abstracted far less U from either seawater or decarbonated seawater than did *Lactobacillus*. Consequently, *Lactobacillus* has great potency in applications to abstract consequential quantities of U from seawater.

15.23 Reduction of Pd(II)

The reduction of soluble Pd(II) to nanoparticulate Pd(0) through enzymatic reactions can be used to abstract Pd from solution, while making a potent catalyst for contaminant remediation (Lloyd et al. 2003). *Desulfovibrio desulfuricans* cells showed the enzymatic Pd(II) reduction to nanoparticulate cell surface bound Pd(0) using H2, pyruvate, or formate as the electron donor. The utilization of H2 as an electron donor, the inhibition of activity by Cu(II), and the periplasmic deposition implicated hydrogenase and possibly cytochrome c3 activity in this reduction. Alongside direct enzymatic Pd(0) synthesis, a novel two-step biologically mediated synthesis technique was outlined (Coker et al. 2010), yielding a nanoscale magnetically recoverable catalyst. Primarily, a biomagnetite carrier was produced by the reduction of ferrihydrite, using cell suspensions of *G. sulfurreducens* (Watts and Lloyd 2013).

15.24 Biotransformation of Cadmium (Cd)

Gram-negative bacteria are more resistant to Cd than Gram-positive ones, and this difference can be attributed to the more complicated cell wall structure of Gram-negative microorganisms (Jjemba 2004). Microbial resistance to Cd is perhaps related to the occurrence of metallothionein proteins which bind and decontaminate several heavy metals. Cd resistance in microbial cells is mainly acquired by active efflux via an energy-dependent mechanism (active convey) to pump out cadmium cations through specific efflux pumps (Jjemba 2004).

15.25 Bioaccumulation and Biosorption Mechanism

Bioaccumulation which is actively involved in heavy metal uptake is a substrate-specific process and requires ATP for its execution (Spain and Alm 2003; Errasquin and Vazquez 2003). Active transport, passive transport and facilitated diffusions are main mechanisms known for metal transport into the bacterial cell. Active transport systems are metal specific, except few exemptions, e.g., same transporters as Zn can transport Cd also (McEldowney 1993). A disadvantage of bioaccumulation is the recuperation of the accumulated metal which has to be done by destructive method which finally causes the damage in the structural integrity of biosorbent (Ansari and Malik 2007).

Change in the pH condition very strongly affects the biosorption (Schiewer and Volesky 2000) (Table 15.8). The various chemical species of a metal found at varying pH values will have different charges and adsorbability at solid-liquid interfaces. pH regulates the speciation and later on solubility of toxic metal ions and also alters the properties of the biomass (Chen et al. 2008). The diverse pH sorption pattern for various heavy metal ions may be associated with the nature of chemical interactions of each metal with biomass (Kiran et al. 2005; Bueno et al. 2008). The level of hydrolysis at diverse pH values varies with each metal, but the usual sequence of hydrolysis involves the formation of hydroxylated monomeric species followed by the formation of polymeric species and consequently the formation of crystalline oxide, which generally get precipitated after aging (Ziagova et al. 2007; Hasan and Srivastava 2009). Being exothermic in nature, the rate of adsorption and ion exchange will increase with an increase in the temperature. However, at high temperatures, a reduction in metal uptake is recorded, as cell walls may be permanently damaged at high temperature. Initial solute concentration has an influence on biosorption, with a higher concentration leading to high solute uptake (Öztürk 2007; Bueno et al. 2008; Uzel and Ozdemir 2009). The dose of a biosorbent has strong impacts on the extent of biosorption. Generally the amount of solute biosorbed increases with an increase in biomass concentration which may be due to the increased surface area of the biosorbent, which in turn increases the number of binding sites (Ziagova et al. 2007; Bueno et al. 2008).

15.26 Heavy Metal Resistance Mechanisms

Mechanisms for the resistance to heavy metals consist of active efflux, reduction, complexation, and sequestration of heavy metal ions from toxic to less toxic state (Nies 1999). These resistance mechanisms are mostly governed by plasmid, which significantly adds to dispersal from cell to cell (Collard et al. 1994, Valls and de Lorenzo 2002), whereas in some bacterial species, resistance mechanism is also related to chromosomes (Spain and Alm 2003; Abou-Shanab et al. 2007). *Cupriavidus metallidurans* CH34 has been reported to have tendency to bioremediate heavy metal-contaminated soils and accumulate selenium (Se) and gold (Au) and volatilize Hg through different reactive processes (Guiné et al. 2003; Sarret et al. 2005; Reith et al. 2006). On exposure of bacterial cells to the high concentrations of heavy metals, the reaction between the metals and different metabolites within cells led to the formation of toxic compounds. Heavy metals enter the bacterial cells through specific mechanisms existing in them, mainly for metal uptake (Ahemad 2012; Spain and Alm 2003). Bacterial cells utilize copper in minute quantities for the synthesis of metabolic enzymes like cytochrome c oxidase, although various bacterial species in soil and water ecosystem are exposed to very high levels of copper as it exists in very high concentration in soil and dihydrogen monoxide because of its wide use in mining, industrial processes, and agricultural practices. So, bacteria have developed numerous types of mechanisms to survive under high copper concentration and copper-induced biotoxicity. Resistance mechanism

Table 15.8 Biosorption by bacterial biomass (mg/g^{-1}) (Ansari et al. 2011)

S. No.	Bacterial species	Metal	pH	Biosorption capacity(mg/g)	References
	Aeromonas caviae	Cd(II)	7	155.3	Loukidou et al. (2004)
	Aeromonas caviae	Cr(VI)	2.5	284.4	Loukidou et al. (2004)
	Aeromonas hydrophila	Pb	5	163.3	Hasan et al. (2009)
	Arthrobacter nicotianae	Th	3.5	75.9	Nakajima and Tsuruta (2004)
	Arthrobacter nicotianae	U	3.5	68.8	Nakajima and Tsuruta (2004)
	Arthrobacter sp.	Cr(VI)	4	9.115	Mishra and Doble (2008)
	Arthrobacter sp.	Cr(VI)	5	175.87	Hasan and Srivastava (2009)
	Arthrobacter sp.	Cu(II)	5	175.87	Hasan and Srivastava (2009)
	B. thuringiensis	Ni(II)	6		Ozturk (2007)
	Bacillus cereus	Pb	NA	36.71	Jian-hua et al. (2007)
	Bacillus cereus	Cu(II)	NA	50.32	Jian-hua et al. (2007)
	Bacillus circulans	Cd(II)	7	26.5	Yilmaz and Ensari (2005)
	Bacillus jeotgali	Zn	7	222.2	Green-Ruiz et al. (2008)
	Bacillus jeotgali	Cd(II)	7	57.9	Green-Ruiz et al. (2008)
	Bacillus licheniformis	U	3.5	45.9	Nakajima and Tsuruta (2004)
	Bacillus licheniformis	Th	3.5	75.9	Nakajima and Tsuruta (2004)
	Bacillus licheniformis	Cr(VI)	2.5	69.4	Zhou et al. (2007)
	Bacillus marisflavi	Cr(VI)	4	5.783	Mishra and Doble (2008)
	Bacillus megaterium	Th	3.5	66.1	Nakajima and Tsuruta (2004)
	Bacillus megaterium	U	3.5	37.8	Nakajima and Tsuruta (2004)
	Bacillus polymyxa IMV8910	U	6	190.4	Shevchuk and Klimenko (2009)
	Bacillus sp.	Hg(II)	6	7.9	Green-Ruiz (2006)
	Bacillus sp. F19	Cu(II)	4.8	89.62	Yan et al. (2008)
	Bacillus subtilis	Th	3.5	74.0	Nakajima and Tsuruta (2004)

(continued)

Table 15.8 (continued)

S. No.	Bacterial species	Metal	pH	Biosorption capacity(mg/g)	References
	Bacillus subtilis	U	3.5	52.4	Nakajima and Tsuruta (2004)
	Bacillus thuringiensis	Cr(VI)	2	83.3	Sahin and Ozturk (2005)
	Chryseomonas luteola	Cr(VI)	4	3	Ozdemir and Baysal (2004)
	Citrobacter freudii	U	NA	48.02	Xie et al. (2008)
	Corynebacterium equi	Th	3.5	71.9	Nakajima and Tsuruta (2004)
	Corynebacterium equi	U	3.5	21.4	Nakajima and Tsuruta (2004)
	Corynebacterium glutamicum	Th	3.5	46.9	Nakajima and Tsuruta (2004)
	Corynebacterium glutamicum	Pb	5	567.7	Choi and Yun (2004)
	Corynebacterium glutamicum	U	3.5	5.9	Nakajima and Tsuruta (2004)
	Desulfovibrio desulfuricans	Pt	2	62.5	de Vargas et al. (2004)
	Desulfovibrio fructosivorans	Pt	2	32.3	de Vargas et al. (2004)
	Desulfovibrio desulfuricans	Pd	2	128.2	de Vargas et al. (2004)
	Desulfovibrio fructosivorans	Pd	2	119.8	de Vargas et al. (2004)
	Desulfovibrio vulgaris	Pt	2	40.1	de Vargas et al. (2004)
	Desulfovibrio vulgaris	Pd	2	106.3	de Vargas et al. (2004)
	Enterobacter sp. J1	Cu(II)	5	32.5	Lu et al. (2006)
	Enterobacter sp. J1	Cd(II)	6	46.2	Lu et al. (2006)
	Enterobacter sp. J1	Pb	5	50.9	Lu et al. (2006)
	Escherichia coli	Cd(II)	5	2.18	Kao et al. (2009)
	Escherichia coli	Cd(II)	5.6–6	10.3	Quintelas et al. (2009)
	Escherichia coli	Fe(II)	2.7–3.5	16.5	Quintelas et al. (2009)
	Escherichia coli	Ni(II)	5.7–6.2	6.9	Quintelas et al. (2009)
	Escherichia coli	Cr(VI)	4.6–5.1	4.6	Quintelas et al. (2009)
	Geobacillus thermoleovorans	Cd(II)	4	38.8	Ozdemir et al. (2009)
	Geobacillus thermoleovorans	Ni(II)	4	42	Ozdemir et al. (2009)

(continued)

Table 15.8 (continued)

S. No.	Bacterial species	Metal	pH	Biosorption capacity(mg/g)	References
	Geobacillus thermoleovorans	Zn	4	29	Ozdemir et al. (2009)
	Geobacillus thermoleovorans	Cu(II)	4	41.5	Ozdemir et al. (2009)
	Geobacillus toebii	Cd(II)	6	29.2	Ozdemir et al. (2009)
	Geobacillus toebii	Ni(II)	4	21	Ozdemir et al. (2009)
	Geobacillus toebii	Zn	5	21.1	Ozdemir et al. (2009)
	Geobacillus toebii	Cu(II)	4	48.5	Ozdemir et al. (2009)
	Micrococcus luteus	U	3.5	38.8	Nakajima and Tsuruta (2004)
	Micrococcus luteus	Th	3.5	36.2	Nakajima and Tsuruta (2004)
	Pseudomonas aeruginosa AT 18	Cu(II)	6.25	86.95	Silva et al.(2009)
	Pseudomonas aeruginosa	Cr(VI)	NA	0.05	Kang et al. (2007)
	Pseudomonas aeruginosa ASU6a	Ni(II)	6	70	Gabr et al.(2008)
	Pseudomonas aeruginosa ASU6a	Pb	7	79	Gabr et al. (2008)
	Pseudomonas aeruginosa AT18	Cr(III)	7.72	200	Silva et al.(2009)
	Pseudomonas aeruginosa PU21	Pb	5	0.7	Lin and Lai (2006)
	Pseudomonas aeruginosaAT18	Zn	7.72	56.4	Silva et al. (2009)
	Pseudomonas fluorescence TEM08	Ni(II)	2	40.8	Uzel and Ozdemir (2009)
	Pseudomonas fluorescence TEM08	Cr(VI)	2	40.8	Uzel and Ozdemir (2009)
	Pseudomonas putida	Pb	5.5	270.4	Uslu and Tanyol (2006)
	Pseudomonas putida	Zn	5	17.7	Chen et al. (2005b)
	Pseudomonas sp.	Cr(VI)	4	95	Ziagova et al. (2007)
	Pseudomonas sp.	Cu(II)	8	0.046	Choudhary and Sar (2009)
	Pseudomonas sp.	Cd(II)	7	278	Ziagova et al. (2007)
	Pseudomonas sp.	Cd(II)	9	0.078	Choudhary and Sar (2009)

(continued)

Table 15.8 (continued)

S. No.	Bacterial species	Metal	pH	Biosorption capacity(mg/g)	References
	Pseudomonas sp.	Ni(II)	8	0.062	Choudhary and Sar (2009)
	Rhodococcus opacus	Cu(II)	6	0.506	Bueno et al. (2008)
	Pseudomonas veronii 2E	Cd(II)	7.5	54	Vullo et al. (2008)
	Rhodococcus opacus	Cr(III)	5	714.29	Bueno et al. (2008)
	Rhodococcus opacus	Ni(II)	5	7.63	Cayllahua et al. (2009)
	Rhodococcus opacus	Pb	5	0.455	Bueno et al. (2008)
	Rhodococcus opacus	Cr(III)	6	1.404	Bueno et al. (2008)
	Shewanella putrefaciens	Zn	NA	22	Chubar et al. (2008)
	Shewanella putrefaciens	Cu(II)	NA	45	Chubar et al. (2008)
	Sphaerotillus natans	Cu(II)	6	60	Beolchini et al. (2006)
	Staphylococcus saprophyticus	Ni(II)	7	16.85	Zamil et al. (2009)
	Staphylococcus saprophyticus BMSZ71	Cu(II)	6	22.36	Zamil et al. (2009)
	Staphylococcus saprophyticus BMSZ71	Cd(II)	7	54.91	Zamil et al. (2009)
	Staphylococcus saprophyticus BMSZ71	Hg(II)	6	78.17	Zamil et al. (2009)
	Staphylococcus saprophyticus BMSZ71	Zn	26.33	7	Zamil et al. (2009)
	Staphylococcus saprophyticus BMSZ71	Pb	5	184.89	Zamil et al. (2009)
	Staphylococcus saprophyticus BMSZ71	Cr(III)	5	22.06	Zamil et al. (2009)
	Staphylococcus sp.	Cr(VI)	1	143	Ziagova et al. (2007)
	Streptomyces coelicolor	Ni(II)	11.1	8	Ozturk et al. (2004)
	Streptomyces coelicolor	Cu(II)	5	66.7	Ozturk et al. (2004)
	Streptomyces rimosus	Fe(II)	NA	122	Selatnia et al. (2004b)
	Streptomyces rimosus	Pb	NA	135	Selatnia et al. (2004c)

(continued)

Table 15.8 (continued)

S. No.	Bacterial species	Metal	pH	Biosorption capacity(mg/g)	References
	Streptomyces rimosus	Cd(II)	8	64.9	Selatnia et al. (2004a)
	T. ferrooxidans	Zn	6	82.6	Liu et al. (2004)
	Thiobacillus ferrooxidans	Cu(II)	6	198.5	Liu et al. (2004)
	Zoogloea ramigera	U	3.5	49.7	Nakajima and Tsuruta (2004)
	Zoogloea ramigera	Th	3.5	77	Nakajima and Tsuruta (2004)

against copper was studied in abundantly found copper-resistant bacteria which were isolated from a copper corroded dihydrogen monoxide distribution system. It was observed that 62% of the total isolates exhibited significant resistance against copper (Lin and Olson 1995). Forty-nine percent of the resistant bacterial isolates had cop or cop-like gene systems as well as both compartmentalization and efflux systems. Plant pathogen *Pseudomonas syringae* showed resistance against copper was due to the distribution of copper accumulated in the cell's periplasm and the outer membrane, and it was resolved that the protective mechanism in *P. syringae* was attributed to four types of proteins (CopA, CopB, CopC, and CopD). All these proteins are encoded by the cop operon found in bacterial plasmid and are found in the periplasm (CopA and CopC), the outer membrane (CopB), and the inner membrane (CopA) and cofunction to sort copper away from bacterial cells (Cooksey 1993). In contradiction, copper resistance system in *E. coli* mainly depends upon efflux mechanism, to prevail copper stress, for which proteins are expressed by plasmid genes *pco*, whose expression depends on chromosomal *cut* genes. Furthermore, two encoded products of genes *cutC* and *cutF* are copper-binding protein and an outer membrane lipoprotein, respectively. Generally bacterial species have attained any one of the above-cited protective mechanisms when exposed to the metal-stressed environment (Spain and Alm 2003). Tolerant capacity to heavy metals of some bacteria is influenced prominently by formation of a bacterial biofilm, which in turn is controlled by quorum sensing molecules (Sarkar and Chakraborty 2008). Each quorum sensing molecule holds at least one heavy metal (Zn) ion as an element of its structure (Hilgers and Ludwig 2001). Heavy metal pollution and antibiotic resistance are also connected and a matter of concern as metal pollutants may also act as co-selective agents for antibiotic resistance in bacteria. Co-selective agents mean when selection for one character simultaneously selects for a secondary character. In this incident, selection for metal resistance also selects for resistance to antibiotics (Baker-Austin et al. 2006). In fact, one explication for the evolution of antibiotic resistance genes, some of which have had extensive evolutionary histories prior to widespread human utilizations of antibiotics, is their competency to function in heavy metal resistance (Aminov and Mackie 2007), essentially preadapting them to current human utilizations of antibiotics. Antibiotic resistance is an earnest threat to human health, and how such resistance evolves and

the role of the environment in this process are of main interest (Baquero et al. 2008). One reason behind this is that antibiotics may degrade in the environment but metals do not, and heavy metal pollution continues to increment (Han et al. 2002). Thus, heavy metal pollution may avail maintain antibiotic-resistant bacterial strains even if input of antibiotics into the environment is reduced. For example, chloramphenicol has been prohibited in China since 1999 (Dang et al. 2008).

Zinc is an essential trace element and biologically not very redox reactive. Therefore, it is not applied in cellular metabolisms like respiration. Though, it is structurally an important component of numerous cellular enzymes. Additionally, it further forms complexes in cells such as zinc fingers in DNA (Spain and Alm 2003). In integration, zinc genuinely exhibits comparatively less toxicity to bacterial cells than other heavy metals, and it usually occurs in high concentrations inside the bacterial cells. Due to this reason, bacteria in heavy metal-contaminated site collect zinc by a prompt but undefined uptake mechanism. Mostly, uptake of zinc ions in bacterial cells is connected with magnesium also, and both ions may be carried out through same mechanism (Cooksey 1993). Bacterial resistance to zinc is generally accomplished via two efflux mechanisms: (I) intermediated by a P-type ATPase effluence system and (II) intermediated by an RND-driven transporter mechanism (Cooksey 1993). In fact P-type ATPase causes catalysis of the reactions via ATP hydrolysis constituting a phosphorylated intermediate, while the RND term belongs to a family of proteins involved in the heavy metal transit. The P-type ATPase effluence system conveys zinc ions across the cytoplasmic membrane by the energy relinquished from ATP hydrolysis. In the same context, a chromosomal gene, *zntA*, was isolated from *E. coli* K-12 and concluded that the gene *zntA* might be responsible for the zinc and other cations conveying ATPase across cell membranes (Beard et al. 1997). In comparison to P-type ATPase effluence system, the RND-driven conveyor system does not derive energy through ATP hydrolysis to convey zinc within the bacterial cells. As an alternate mechanism, it is power-driven by the proton gradient across the cell wall especially in Gram-negative bacteria (Cooksey 1993; Spain and Alm 2003). Numerous metal-resistant bacteria have been already reported. Conjugal transfer of the *A. eutrophus* genes encoding plasmid-borne resistance to cadmium, cobalt, and zinc (*czc* genes) from *E. coli* to *Alcaligenes eutrophus* was investigated under in vitro culture and in the soil samples. In the donor strain, *E. coli*, czc gene did not express, but expressed in the recipient strain, *A. eutrophus*. Henceforth, expression of heavy metal resistance genes in the bacteria cultured on a medium containing heavy metals indicates escape of the *czc* genes. The two plasmids, nonconjugative, mobilizable plasmid pDN705 and the nonconjugative, nonmobilizable plasmid pMOL149, were used for the cloning of DNA fragment. Results demonstrated that in certain soils, environmental conditions and specifically nutrient levels are helpful to gene transfer (Top et al. 1990).

Isolation and characterization of three copper-resistant, Gram-negative bacteria have been reported. Among them, *Alcaligenes denitrificans* AH abode the highest copper concentration (MIC = 4 mM $CuSO_4$). All three strains exhibited different levels of resistance to other metal ions. Genetic makeup of *A. denitrificans* AH consists of cross-hybridization product of the *mer* (mercury resistance) determinant of

Tn2l and the *czc* (cobalt, zinc, and cadmium resistance), *cnr* (cobalt and nickel resistance), and *chr* (chromate resistance) determinants of *A. eutrophus* CH34. The second strain, revealed as *Alcaligenes* sp. strain PW, carries czc, cnr, and mer homologs on the 240-kb plasmid pHG29-c and a chr determinant on the 290-kb plasmid pHG29-a; a third large plasmid, pHG29-b, length 260-kb is cryptic. In comparison to the *Alcaligenes* strains, which were sequestered from metal-contaminated dihydrogen monoxide, *Pseudomonas paucimobilis* CD strain was sequestered from the air. *P. paucimobilis* CD strain harbors two cryptic plasmids of 210-kb and 40-kb pHG28-a and pHG28-b plasmid, respectively. No homology was observed between the metal ion resistance determinants of *A. eutrophus* CH34 and *P. paucimobilis* CD as confirmed through southern analysis (Dressler et al. 1991). An entire range of 272 *Pseudomonas* spp. and 161 *Bacillus* spp. strains were isolated from sample collected from industrial waste soils at 4 different locations. The maximum tolerable metal concentrations (MTCs) of Cr, Cu, Ni, Co, Cd, and Zn for each isolate were resolved. 73.9% of the total bacterial strains, isolated from soil samples, showed resistance to Cr, while 26%, 18.4%, 11.5%, 9.2%, and 7.3% of the isolates displayed resistance to Ni, Zn, Cd, Co, and Cu, respectively (Sevgi et al. 2010). For each metal, microorganisms have developed a specialized or a set of resistance mechanisms. The proficiency of these mechanisms is influenced by many parameters, such as the metal itself, the species under study, time, temperature, pH, occurrence of plant groups near the microfauna, and most significantly the interfaces of the metal with other compounds. Complete analysis of some *Rhizobiales* in heavy metal-contaminated soils suggests that these soil organisms are liable to bear systems to survive in highly toxic habitats (Cánovas et al. 2003). Members of the *Caulobacter*, *Sphingomonas*, and *Rhizobium* families sequestered from mine tailings may be accountable for the reduction and mobilization of arsenic (Macur et al. 2001). These mechanisms of metal resistance are frequently associated with convey-related membrane proteins that mediate bacterium's direct metabolic interactions with the intricate soil and aquatic environments in which they inhabits. Cells provoke a variety of ABC conveyor proteins that shuttle numerous molecules across the plasma membrane to maintain the integrity of the intracellular milieu. Consequently, the analysis of *Sinorhizobium meliloti*, *Mesorhizobium loti*, and *Rhizobium leguminosarum* convey proteins is an important step to sort out the methodologies evolved by this microorganism to habituate to toxic environmental conditions (Sá-Pereiraa et al. 2010).

15.27 Conclusion

Heavy metal contamination of soil, by means of industrial wastes, sewage inflow, contaminated groundwater, etc., can induce serious problems to soil, cropping, vegetation, and in turn human health. Heavy metal accumulation by plant tissues, its presence in the soil sedulously, or its presence in groundwaters is not a salubrious sign for the environment. So cleaning of our environment from these pollutants, such that they are no longer toxic to life, is of great paramountcy. Investigation of

the microbe-metal interactions provides insight into the potential of microorganisms to alter toxicity of heavy metals and radionuclides and to influence their comportment in the environment. Understanding the underlying mechanisms of these interactions is paramount for the development of bioremediation strategies as well as the utilization of microbes and their bio-components as templates for the formation of metallic nanoparticles with industrial applications. Numerous approaches have been studied for the development of more frugal and efficacious metal sorbents, such as microbial cells. However, microbial biomass in its native form is not felicitous for astronomically immense-scale process utilization.

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Bioremediation of Nutrients and Heavy Metals from Wastewater by Microalgal Cells: Mechanism and Kinetics

16

Vishal Singh and Vishal Mishra

Abstract

Conventional chemical and physical treatment methods applied for the treatment of wastewater tend to be complicated, energy demanding and expensive. Biological waste treatment process involving microalgae provides an economical, sustainable and alternate means of advanced wastewater treatment process coupled with simultaneous recovery of nutrients and manufacture of commercially valuable products like single-cell protein, biofuel, etc. Updated information regarding the advancements made in the treatment process, mechanism and kinetic models involved in nutrient removal by microalgae are provided in this review. Advancements such ultrasonic treatment, use of algal-bacterial symbiosis system, blending of two different wastewater and use of photo-sequencing batch bioreactors for the treatment of municipal, domestic, livestock and industrial wastewater are discussed in brief. The present work focuses mainly on the primary mechanisms involved in the assimilation of nitrogen, carbon and phosphorus inside the microalgal cell. Not only a brief description of metal-ion uptake by processes such as ion exchange, complex formation, precipitation and physical adsorption and role of the plasma membrane, cell wall, vacuoles, chloroplast and mitochondria is discussed in this investigation, but also the various kinetic models of nutrient removal such as Stover-Kincannon, Michaelis-Menten, Gompertz model and Luedeking-Piret model with their experimental curve fitting results obtained from microalgal cell-mediated treatment process are also discussed.

Keywords

Wastewater treatment · Microalgae · Nutrient removal · Heavy metal · Bioremediation · Kinetics

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BOD	Biological oxygen demand
Cd2+	Cadmium ion
Co	Cobalt
CO_2	Carbon dioxide
COD	Chemical oxygen demand
Conc.	Concentration
Cr2O72-	Chromate ion
Cr3+& Cr6+	Chromium ions
Cu2+	Copper ion
DW	Domestic wastewater
Fe3+	Ferrous ion
H_2O	Water molecule
$H_2PO_4^-$	Dihydrogen phosphate
HCO_3^-	Bicarbonate
Hg2+	Mercury ion
HMs	Heavy metal
HPO_4	Hydrogen phosphate
HRT	Hydraulic retention time
N	Nitrogen
NADP	Nicotinamide adenine dinucleotide phosphate
NH_4^+-N	Ammonium nitrogen
Ni2+	Nickel ion
NO_3^--N	Nitrate nitrogen
O_2	Oxygen
P	Phosphorus
Pb2+	Lead ion
PO_4^{3-}-P	Phosphate phosphorus
RE	Removal efficiency
RuBisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
Temp.	Temperature
TKN	Total Kjeldahl nitrogen
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
Zn2+	Zinc ion

16.1 Introduction

The major problems which the world is facing in the twenty-first century are the increase in the level of pollution and the demand for energy. According to the 2017 report of United Nations World Water Development, 2212 km^3 per year effluent is discharged in the environment from municipal, agriculture and industrial sources (Koncagul et al. 2017). Eighty percent of this polluted water is released into the waterbodies without appropriate treatment. Tribulations of downstream eutrophication and ecosystem damage occur due to the discharge of untreated sewage containing an excess of nitrogen and phosphorus (Correll 1998). Some adverse effects of eutrophication include the development of algal blooms, low dissolved oxygen, loss of critical species and undesirable pH shift resulting in degradation of the freshwater ecosystem (Renuka et al. 2013). Physical and chemical techniques are conventionally available for the remediation of wastewater and nutrient removal, but they are not economical as they consume a significant amount of energy and chemicals (Franzini et al. 1980). Production of secondary chemical sludge occurs during chemical treatment making it unsuitable for safe disposal (Hoffmann 1998).

In comparison with the conventional treatment processes, biological treatment using microalgae efficiently performs the removal of nutrients in a cost-effective and eco-friendly mode with the additional advantages of recovery of resources (e.g. recovery of nutrients) and recycling (Oswald 2003). Application of microalgae in wastewater treatment is notably fascinating because of its photosynthetic capabilities, i.e. it converts solar energy and CO_2 into beneficial biomass and exploits substrates such as ammonium nitrogen and phosphate (de la Noue and de Pauw 1988). In fact, high removal efficiencies (approximately 80–100%) of phosphorus and nitrogen from wastewater discharged from various sources (e.g. municipal, agriculture and industrial) have been specified for microalgae (Gonzalez et al. 1997; Phang et al. 2000; Li et al. 2011; Sydney et al. 2011; Zhu et al. 2013). Advantages which microalgae offer when applied for wastewater treatment are as follows: (i) the microbial biomass produced can be used for production of protein supplement (single-cell protein), animal feed and biofuel, (ii) production of fertilizers from microbial biomass, (iii) evolution of oxygen during photosynthesis (Rawat et al. 2011; Renuka et al. 2013).

With these facts, the present literature review focuses upon the treatment of wastewater by microalgae grown in varying environmental conditions. In the sideline of the work, the current investigation also provides deep insight into the primary mechanism and kinetics of pollutant degradation/remediation mediated by microalgal cells.

16.1.1 Microalgae

Microalgae are characterized as a microscopic photosynthetic organism. They are placed in the category of thallophytes, i.e. lacking leaves, stem and roots. Their main photosynthetic pigment is chlorophyll a. They exist both in marine and

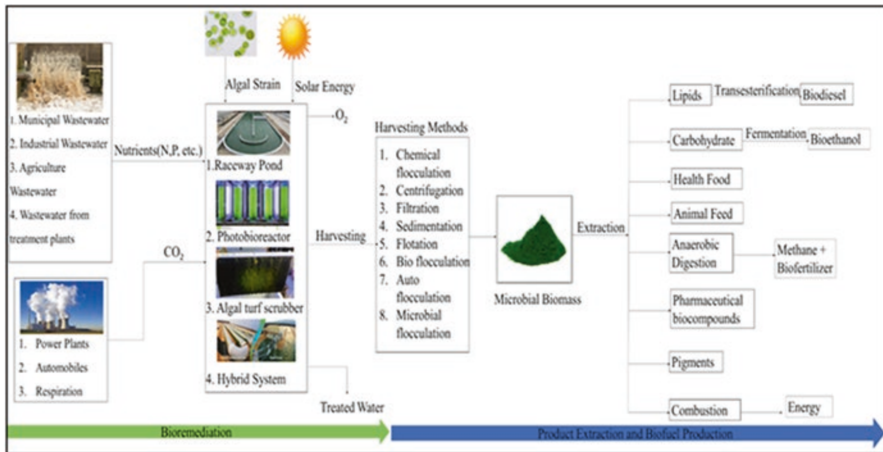


Fig. 16.1 Algae-mediated wastewater treatment with CO_2 sequestration coupled with nutrient recovery

freshwater habitat in the form of single cells, chains and flocs (Falkowski and Raven 1997). Their growth is operated by almost similar photosynthetic process adapted by higher species of the plant kingdom, i.e. converting solar energy and CO_2 into microalgal biomass (Metting and Pyne 1986; Pelczar and ChanECS 1993). They naturally grow as a suspension culture in water and relatively with faster growth rates. In microalgal cells, there is no requirement of the vascular system for the transportation of nutrients, and each cell is capable of directly uptaking dissolved nutrients (Pelczar and ChanECS 1993). Microalgae can be easily genetically manipulated and exploited in mass culture for production of biomass and carbon sequestration from the air (Mitra and Melis 2008). They can survive for a prolonged period of time and can acclimatize in varying environmental conditions. This is due to the fact that microalgal cells can form a resting cyst, which remains in a dormant condition during unfavourable environment (Rengefors et al. 1998).

Figure 16.1 depicts an illustration of integrated microalgae cultivation-wastewater treatment-harvesting-biofuel production. Figure 16.1 demonstrates that microalgae are grown by utilizing the nutrients from wastewater and CO_2 emerging out from industrial combustion processes or during respiration. Finally, microalgal biomass is harvested and can be used for the production of energy and protein supplements, biofertilizer, etc. (Razzak et al. 2013).

16.2 Wastewater Sources Treated Using Microalgae

The characteristics and composition of wastewater differ depending on the origin of discharge. It contains the different nutrients essential for microalgal growth (Komolafe et al. 2014). Table 16.1 reports the different microalgae species used in the wastewater treatment.

It is clear from Table 16.1 that the successful microalgae cultivation has been done in municipal, industrial and agricultural wastewater. Table 16.1 also reports that *Chlorella* sp. is the main algal species which has been widely studied. The rationale behind the massive involvement of microalga *Chlorella* is its ability to remove the nutrients with removal efficiency up to 90%.

16.2.1 Domestic Wastewater

Wastewater discharged from the residences and commercial locations constitute the domestic wastewater (DW). Other sources include public and private institutional facilities. Chemical constituents of DW include both organic (carbohydrates, fat, oil, protein, surfactants, etc.) and inorganic (nitrogen, phosphorus, sulphur, etc.) components (Metcalf and Eddy 2014). Ren et al. (2019) have inspected the effect of ultrasonic treatment for RE of nutrients from non-sterile domestic effluent. Ultrasonic waves reduce the reaction time and improve mass transfer. Authors cultivated *Scenedesmus sp.* in bubble column reactor made up of polymethyl methacrylate (600 ml working volume) using DW as medium for 7 days, and ultrasonic waves of different frequencies (ranging from 0 to 30 kHz), power (ranging from 0 to 50 W) and time interval (ranging from 0 to 50 min) were exposed on the reactor. Maximum removal of TP and TN reached up to 97.7% and 96.8%, respectively, at the optimal ultrasonic treatment parameters: power, 20 W; frequency, 18kHz; and time, 10 min (Ren et al. 2019).

In another study, sequencing batch biofilm reactor (SBBR) was modified by the incorporation of microalgae to construct an algal-bacterial symbiosis (ABS) system for enhancing the RE of nutrients from DW. SBBR was made up of glass (4 L working volume) with ceramic carriers fitted at the upper side of the reactor that was favourable for both the ABS system development and algae enhancement. This ABS system improved the removal efficiency of TN from 38.5% to 65.8% and of TP from 31.9% to 89.3% (Tang et al. 2018). Enhancement of nutrient removal efficiencies is also done by using immobilization techniques. Katam and Bhattacharyya (2019) compared the removal efficiency of two systems: (i) system A (suspended activated sludge and immobilized mixed microalgal culture system) and (ii) system B (suspended co-culture system). They immobilized the microalgal consortium in alginate polymer. System A showed relatively higher removal of nitrogen and phosphorus (91% and 93%) compared to system B (58% and 80%) (Katam and Bhattacharyya 2019).

16.2.2 Municipal Wastewater

Extensive investigation has been done to estimate the potential and prospective application of microalgae for municipal wastewater treatment (Li et al. 2011; Zhou et al. 2012b). Municipal wastewater contains a varying amount of domestic

Table 16.1 Different sources of wastewater treated by different microalgae species

Wastewater source	Microalgal species	Cultivation method/ harvesting method	Percentage (%) of N[a] removed	Percentage (%) of P[b] removed	Biomass yield (Y)/ product yield (P)	Reference
85–90% carpet industry wastewater blended with 10–15% municipal sewage	Mixed culture of 15 native isolated algae species	Raceway ponds (750 L)/ centrifugation	>96% TN[c]	>96% TP[d]	$P = 0.039\ gL^{-1}d^{-1}$	Chinnasamy et al. (2010)
Anaerobic centrate	*Chlorella sorokiniana*	Erlenmeyer flask (1 L)/na	94.29 NH_4^+_N[e]	83.3 PO_4^{3-}-P[f]	$P = 77.14\ mgL^{-1}d^{-1}$	Ramsundar et al. (2017)
Anaerobic digester effluent	LLAI (resembles *Chlorella* sp.)	1 L spinner flask/filtration (0.45 µm syringe filter)	>90% NH_4^+_N	>90% PO_4^{3-}-P	$P = 0.34\ g\ L^{-1}d^{-1}$	Wahal and Viamajala (2016)
Anaerobic digested starch and alcohol wastewater	*Chlorella pyrenoidosa*	Conical flasks (2 L)/ centrifugation	91.64 TN	90.74 TP	$Y = 3.01\ gL^{-1}$	Yang et al. (2015)
Anaerobic membrane bioreactor effluent	*Chlorophyceae* and *Cyanobacteria*	Photobioreactor (10 L)/ membrane filtration 2(0.45 mm)	67.2 NH_4^+-N	97.8 PO_4^{3-}-P	P = 234 mg/L/d	Ruiz-Martinez et al. (2012)
Autoclaved concentrated municipal wastewater	*Auxenochlorella protothecoides* UMN280	Erlenmeyer flask (2 L), 1 L Roux culture bottle bioreactor/self-sedimentation, centrifugation	59 TN	81 TP	P = 269 mg/L/d	Zhou et al. (2012a)
Aquaculture wastewater	*Ankistrodesmus falcatus*	1 L conical flask/ centrifugation	86.45 NH_4^+_N 80.85 NO_3-N[g]	98.52 PO_4^{3-}-P	P = 160.79 mg/L/d	Ansari et al. (2017)

Autoclaved piggery wastewater	*Chlorella zofingiensis*	Photobioreactor (1.37 L)/ centrifugation and nylon membrane filter	82.70 TN	98.17 TP	P = 296.16 mg/L/d	Zhu et al. (2013)
Brewery industry	*Chlorella vulgaris* UTEX-265	Erlenmeyer flasks (500 ml)/ auto flocculation and centrifugation	>90 TN	>90 TP	Y = 3.20 gL^{-1}	Farooq et al. (2013)
Cellulosic ethanol wastewater	*Chlorella vulgaris* CEW-1	2 L photobioreactor/ centrifugation	97.6 TN	84.2 TP	Y = 1.98 gL^{-1}	Li et al. (2017)
Dairy manure	*Neochloris oleoabundans*	Erlenmeyer flasks (250 ml) and 50 L	90–95 TN	Na	P = 44–88 $mgL^{-1}d^{-1}$	Levine et al. 2011
		Photobioreactor/ centrifugation				
Dairy wastewater	Microalgae consortia	Photobioreactor (400 ml)/ filtration (0.45 μm nylon membrane)	Na	TP 91.16–95.96	730.4–773.2 $mgL^{-1}d^{-1}$	Qin et al. 2016
Defoamed wastewater	*Desmodesmus* sp.	20 L 3 open batch reactors	80 TN	38 PO_4^{3-}-P	Y = 0.58 gL^{-1}	Komolafe et al. 2014
		1.Ozone floatation with centrifugation			P = 0.013 $gL^{-1}d^{-1}$	
		2. Sedimentation with centrifugation				
Domestic anaerobic digester centrate	*Chlorella sorokiniana*	Photobioreactor (1 L Duran bottle)/centrifugation	100 TN	Na	Y = 320 mg/L	Lizzul et al. 2014
Domestic wastewater	*Botryococcus terribilis*	1 L Erlenmeyer flasks/ centrifugation	Na	Na	P = 282 mg/L/d	Cabanelas et al. (2013)
Hydroponic cultivation of tomatoes	*C. vulgaris*	Plexiglass tank/ centrifugation	Nitric N – 99	Soluble-P – 94	Na	Baglieri et al. (2016)
			NH_4^+_N-83	TP – 94		
	Scenedesmus quadricauda	Plexiglass tank/ centrifugation	Nitric N – 99	Soluble-P – 88	Na	Baglieri et al. (2016)
			NH_4^+-N	TP – 89		

(continued)

Table 16.1 (continued)

Wastewater source	Microalgal species	Cultivation method/ harvesting method	Percentage (%) of N[a] removed	Percentage (%) of P[b] removed	Biomass yield (Y)/ product yield (P)	Reference
Industrial centrate	*Chlamydomonas reinhardtii*	Erlenmeyer flasks and biocoil photobioreactor/ flocculation	83 TN	14.45 TP	Y = 2.0 $gL^{-1}d^{-1}$	Kong et al. (2010)
Industrial riboflavin effluent	*Chlorella pyrenoidosa*	Thermostatic culture box/ centrifugation	78.76 TN	94.78 TP	Y = 1.25 gL^{-1}	Sun et al. (2013)
Industrial wastewater	*Chlamydomonas* sp. TAI-2	Photobioreactor (6 L)/ centrifugation	100 NH_4^+-N 100 NO_3-N	33 TP	Y = 1.5 gL^{-1}	Wu et al. (2012)
Municipal wastewater	*Chlorella vulgaris*	2 L Erlenmeyer flasks/ filtration	100 NH_4^+-N 82 NO_3-N	Na	P = 138.76 $mgL^{-1}d^{-1}$	Ebrahimian et al. 2014
NaClO-pretreated piggery wastewater	*Chlorella zofingiensis*	Photobioreactor (1.37 L)/ centrifugation and nylon membrane filter	84.49 TN	95.26 TP	P = 285.96 $mgL^{-1}d^{-1}$	Zhu et al. (2013)
Potato processing wastewater	*Chlorella sorokiniana*	Photobioreactor (5 L)/ sedimentation	>95 TN	80.7 soluble P	P = 18.8 $mgL^{-1}d^{-1}$	Hernandez et al. (2013)
Recirculating aquaculture system	*Synechocystis sp.*	Acrylic photobioreactor	Na	98.6 ± 6% TP	Na	Rojsitthisak (2017)
Soybean processing wastewater	*Chlorella pyrenoidosa*	500 ml conical flasks/ centrifugation	89.1 NH_4	70.3 TP	P = 0.64 $gL^{-1}d^{-1}$	Hongyang et al. (2011)
Synthetic wastewater	*Chlorella vulgaris*	250 ml cylindrical glass column/ centrifugation	89.4 TN	91.4 TP	Na	Xu et al. (2016)

[a]Nitrogen
[b]Phosphorus
[c]Total nitrogen
[d]Total phosphorus
[e]Ammonium nitrogen
[f]Phosphate
[g]Nitrate

(80–95%) and industrial (5–20%) influents, which broadly depends upon the local activities. It includes inorganic elements such as ammonia and phosphates which support microalgae growth, as well as contain micronutrients such as coppers and magnesium required for their growth (Guldhe et al. 2017). Successful cultivation of *Chlorella* sp. was carried out in wastewater samples obtained from four different locations from the municipal wastewater treatment plant. Authors collected samples (i) before primary treatment, (ii) after primary treatment, (iii) wastewater from activated sludge chamber and (iv) centrate (wastewater generated during centrifugation of sludge). Removal efficiency (RE) for PO_4^{3-}-P, COD and NH_4^+-N was obtained in the range of 78.3–82.4%, 83.2–90.6% and 50.9–83.0%, respectively (Wang et al. 2010). In another study, *Chlorella sorokiniana* was grown in influent coming to the treatment plant and anaerobic tank centrate under both heterotrophic and mixotrophic cultivation mode. Effective NH_4^+-N (94.29%) and PO_4^{3-}-P (83.30%) removal was obtained under mixotrophic condition using anaerobic tank centrate (Ramsundar et al. 2017). Zhai et al. (2017) employed the response surface methodology (RSM) technique for the prediction of the optimal conditions to increase the RE of the substrate by *Spirulina platensis* from synthetic simulated municipal wastewater, confirmed by conducting laboratory experiment. The optimal parameters for growth were light intensity of 3300–3400 lx and pH 8.8–8.9 at 25 ± 1 °C with aeration rate at 0.5 vvm, and the daily illumination time was fixed to 12 h. The removal efficiency obtained under the optimum conditions was 81.51% for nitrogen and 80.52% for phosphorus (Zhai et al. 2017).

Photo-sequencing batch bioreactors were used for the treatment of municipal discharge with mixed co-culture of microalgae and bacteria. The RE of 98 ± 2% for TKN and 87 ± 5% for COD was obtained without forced aeration, and the combined effects of microbial oxygen consumption and photosynthetic oxygenation were studied (Foladori et al. 2018). For reducing the cost of cultivation of microalgae at larger scale, *Chlorella zofingiensis* was cultivated in municipal wastewater by supplementing it with pig biogas slurry. Batch experiments were conducted with different proportions of municipal wastewater and pig biogas slurry. The results exhibited that, when 8% of pig biogas slurry was supplemented in municipal wastewater, it had remarkable effects on microalgae growth with 93% TN and 90% TP removal (Zhou et al. 2018).

16.2.3 Industrial Wastewater

Industrial wastewater characteristics and composition vary from one dumping site to another and include a high concentration of decomposable and non-decomposable inorganic and organic materials and growth inhibitory constituents depending upon the type of industry (Razzak et al. 2013; Guldhe et al. 2017). Several researches have been published in recent time which demonstrate the successful treatment of effluents discharged from textile industry, carpet mill, olive mill, palm mill, pork processing industry, sugar mill, tannery industry, etc. by microalgae (Di Iaconi et al. 2010; Zhou et al. 2014; Chen et al. 2015; Wu et al. 2017; Hülsen et al. 2018). Textile

effluent is composed of different organic materials, phosphorus and nitrogen, that can be utilized for microalgae cultivation. The influence of pH and various sources of phosphorus and nitrogen was investigated during the cultivation of *Chlorella* sp. G-23 in varying dilutions of textile wastewater. The highest NH_4^+-N RE was 78 ± 3% at 0% dilution rate and 84 ± 4% at 10% dilution rate with aeration at pH 9. For COD, peak RE (>60%) was obtained at 0% dilution rate, without any effect of aeration. There were no notable effects of the type of nitrogen source on microalgae growth (Wu et al. 2017). In another study, textile wastewater was treated by mixed microalgae consortia (*Chlorella* and *Scenedesmus* sp.) in a fed-batch reactor. The fed-batch reactor was operated for five cycles, and the duration of the cycles was reduced (30–10 days) as the cycles were repeated. This led to the gradual adaption of microalgae in textile wastewater. RE of 70% of total nitrogen and 95% of total phosphorus was obtained throughout the operation (Kumar et al. 2018).

Tannery wastewater is rich in nitrogenous compounds and is also composed of high carbon-based content which supports microalgae growth in both autotrophic and heterotrophic mode. da Fontoura et al. (2017) used different concentrations (20–100%) of tannery wastewater for the cultivation of *Scenedesmus* sp. at different light intensities (20–200 μmol photons $m^{-2}s$) at temperature 25 °C and with constant aeration. The maximum RE of COD (80.33%), NH_4^+-N (85.63%) and PO_4^{3-}-P (96.78%) was obtained at a light intensity of 102.5 μmol photons $m^{-2}s$ and wastewater concentration of 88.4% (da Fontoura et al. 2017). During the cultivation of purple phototrophic bacteria (PPB) and microalgae in tannery wastewater, microalgae showed better removal efficiency. Both microalgae and PPB were cultivated on five different agro-industrial wastewater (poultry, pork, dairy, red meat and sugar processing industry). PPB showed moderate removal (up to 80% NH_4^+-N, 55% PO_4^{3-}-P, 74% COD), whereas microalgae showed higher RE (up to 91% NH_4^+-N, 73% PO_4^{3-}-P, 91% COD) (Hulsen et al. 2018).

16.2.4 Livestock Effluent

Animal feeding operations generate a tremendous amount of dung and manure-contaminated wastewater. Recently, livestock processes are carried out at a larger scale, thus generating a large amount of effluent (Kellogg et al. 2000). Livestock effluent is often rich in ammonium, organic phosphorus and nitrogen, thus providing vital nutrients for supporting microalgae growth (Wang and Lan 2011; Zhou et al. 2012a; Mobin and Alam 2014). In a study, *Botryococcus braunii* was cultivated in submerged membrane photobioreactor (SMPBR), and its ability was investigated to conduct tertiary treatment of livestock effluent. Semi-continuous photobioreactor was operated in three phases based on the hydraulic retention time (HRT) (3, 4 and 5 days) to evaluate nutrient removal efficiency. Results showed that shorter HRT (3 days) provided better removal efficiency of TP (85%) and TN (96%) (Lee et al. 2018). Five microalgal species *C. vulgaris* (FACHB-1227), *Parachlorella kessleri* TY, *S. obliquus* (FACHB-417), *S. quadricauda* (FACHB-1468) and *Chlorococcum* sp. GD were cultivated in cattle farm wastewater without dilution. *C.*

vulgaris showed the highest nutrient removal efficiency which was 98.69%, 81.16%, 83.59%, 85.29% and 62.30% for NO_3^--N, NH_4^+-N, NO_2^--N, TP and COD, respectively (Lv et al. 2018).

Piggery wastewater (PWW) is categorized as one of the most polluted wastewater due to its high values of BOD and COD because of the presence of high concentration of organic nitrogen and organic matter (Olguin 2012). The applicability of microalgae for the treatment of PWW can prove to be an economical and effective way for the assimilation of nutrients, organic matter and emerging pollutants. A comparative investigation was performed to evaluate the efficiency of algal-bacterial photobioreactors treating PWW under outdoor and indoor conditions. Four algal-bacterial photobioreactors (each with 3 L and without closing lid) were operated under outdoor and indoor conditions for the treatment of diluted (10 and 20 times) PWW for 4 months and 26 days of HRT. The highest RE for TOC and TP (94 ± 1% and 100%, respectively) was observed under indoor conditions for 10 times dilutions, while the highest RE for TN (72 ± 1%) was obtained under outdoor conditions for 10 times dilutions. *Chlorella vulgaris* and *Proteobacteria* were the dominant species in the aforesaid removal (Garcia et al. 2017). For improving the nutrient RE, the blending of PWW is carried out with other wastewaters. *Chlorella vulgaris* was cultivated for 7 days in three different mixtures of PWW with brewery wastewater: (i) PWW and malt processing wastewater, (ii) PWW and brewing (saccharifying and fermenting) processing wastewater and (iii) PWW and packaging processing wastewater. The mixture of PWW and packaging processing wastewater showed the maximum RE for ammonia (100%), TN (96%), TP (90%) and TOC (93%) at pH 7.0 and mixing ratio 1:5 (Zheng et al. 2018). In another study, PWW and winery wastewater were mixed in the ratios of 20:80, 50:50, 80:20, 100:0 and 0:100. Mixtures were then inoculated with the soil microalga *Chlorella* sp. MM3 and were grown for 10 days. Mixture of 20:80 showed the maximum removal efficiency which was 100% for NH_3-N, 96% for TN, 90% for TP and 93% for COD, thus proving it to be an effective approach for phycoremediation of the mixture of piggery and winery wastewater (Kumar et al. 2018).

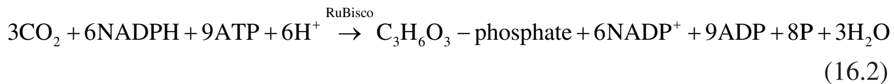
16.3 Mechanism of Nutrient Removal

16.3.1 Carbon Assimilation

Carbon dioxide (CO_2) is one of the critical atmospheric pollutants that contribute to the significant level of greenhouse gas. The rapid development of the industries and urban areas is considered to be a significant source of inorganic carbon (Razzak et al. 2013). Photosynthesis mediated by microalgae represents one of the practical approaches for CO_2 fixation (Yanagi et al. 1995; Wang et al. 2008). Microalgal biomass is nearly composed of 50% carbon of its total weight (in %) (Sanchez Miron et al. 2003). Microalgae utilize carbon in either autotrophic mode or heterotrophic mode. Figure 16.2 represents the flow diagram of carbon assimilation and fatty acid biosynthesis in both autotrophic and heterotrophic cultivation mode.

In the autotrophic mode, CO_2 is fixed through light and dark reaction (Calvin cycle) by using light energy and water molecules with the simultaneous release of oxygen (Campbell et al. 2006; Williams and Laurens 2010). The schematics have been shown in Eqs. (16.1) and (16.2) (Raven et al. 1999):

$$2H_2O + 3ADP + 2NADP^+ + 3P \xrightarrow{\text{light}} 2H^+ + 3ATP + 2NADPH + O_2 \quad (16.1)$$



$$3CO_2 + 6NADPH + 9ATP + 6H^+ \xrightarrow{\text{RuBisco}} C_3H_6O_3 - \text{phosphate} + 6NADP^+ + 9ADP + 8P + 3H_2O \quad (16.2)$$

In the heterotrophic mode, microalgae utilize organic carbon sources. Figure 16.2 shows how organic carbon or the carbon fixed during photosynthesis is used for the synthesis of polysaccharide, precursor fatty acid and other hydrocarbons. Synthesized fatty acids are then translocated to the smooth endoplasmic reticulum and converted into triacylglyceride, and it buds off into oil bodies in the cytosol (Scott et al. 2010). The requirement of CO_2 is also for the maintenance of the pH of the medium (Salama et al. 2017).

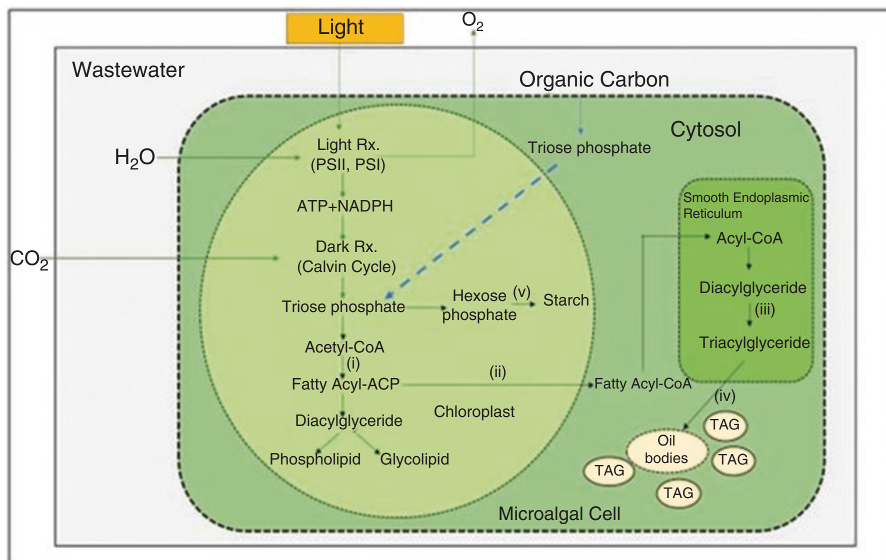


Fig. 16.2 Flow diagram for carbon capture and lipid biosynthesis by microalgae. The green line represents the autotrophic mode, and the blue dotted line represents the heterotrophic mode. Number indicates (i) FAS (fatty acid synthase) and ACCase (acetyl-CoA carboxylase); (ii) acyl-CoA synthetases and fatty acid thioesterases; (iii) triacylglyceride (TAG) biosynthesis enzymes, including acyl-CoA:DGAT (diacylglycerol acyltransferase); (iv) pathway of formation of oil bodies; and (v) starch synthase and ADP-glucose pyrophosphorylase

Additionally, microalgae can also assimilate soluble carbonates for their carbon requirements. When the medium pH is low (5–7), microalgal cells uptake CO_2 through diffusion. At high pH (more than 7), bicarbonate (HCO_3^-) form of carbon is present in the solution. It is transported into the cells through active transport by the activity of external carbonic anhydrase (Sayre 2010; Picardo et al. 2013; Sydney et al. 2014).

16.3.2 Assimilation of Nitrogen

Microalgae require nitrogen (N) as one of their essential elements for growth, which can be easily obtained from wastewater in an abundant amount (Wang et al. 2010). It is present in various biological macromolecules including enzymes, proteins, genetic materials (DNA/RNA) and energy transfer units (ATP/ADP). Microalgae assimilate inorganic N (including NH_4^+, NO_3^- and NO_2^-) and convert them to organic N. Cyanobacteria also transform atmospheric nitrogen into ammonia through a process named nitrogen fixation (Vymazal 2007; Cai et al. 2013). Figure 16.3 shows the pathway for the assimilation of inorganic nitrogen by microalgae.

It became evident from Fig. 16.3 that nitrate assimilation is carried out in two transport pathways mediated by two steps of reduction. Initially, nitrate is translocated in the cytosol through the plasma membrane. Then, nitrate is reduced to nitrite in a reduction reaction catalysed by nitrate reductase (NR) present in the cytosol. It takes two electrons from NADH (nicotinamide adenine dinucleotide – present in reduced form) and transfers it to nitrate. Nitrite is then translocated into the chloroplast, where its reduction to ammonium occurs in a reduction reaction catalysed by nitrite reductase (NiR) by the transfer of six electrons from a reduced form of ferredoxin (Fd). Finally, glutamate synthetase catalyses the merging of ammonium

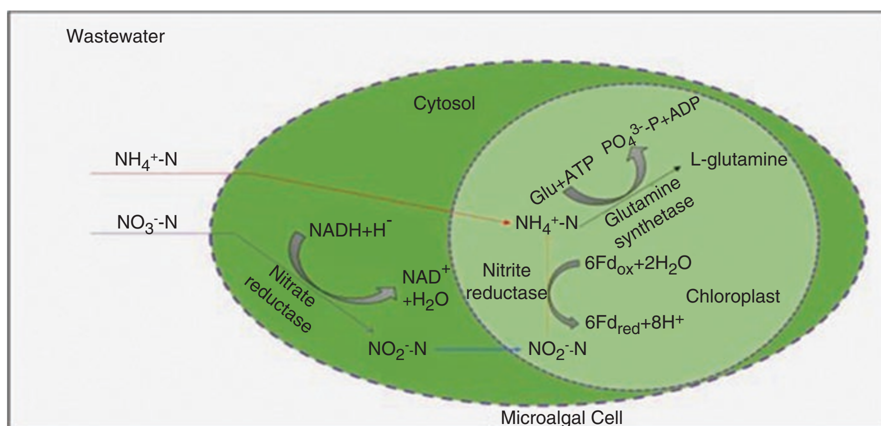


Fig. 16.3 Diagrammatic representation of inorganic nitrogen assimilation by microalgae

into amino acid glutamine by using adenosine triphosphate and glutamate (Sanz-Luque et al. 2015). Therefore, all types of inorganic N are first reduced to ammonium N before merging into amino acids within the cell (Salama et al. 2017). It was reported that when glutamate was added to the wastewater, it caused 70% further reduction of NH_4^+ by each cell of *Chlorella vulgaris* (Khan and Yoshida 2008).

16.3.3 Assimilation of Phosphorus

Microalgal cells also require phosphorus for their growth, and it plays a vital role in controlling its biomass composition in fresh water. It is an integral part of the DNA, RNA, ATP, protein/amino acids and lipids/fatty acids present in the cell wall. It also occurs in intermediates of carbohydrates and fatty acid metabolism and cell membrane materials (Cembella et al. 1984). Absence or depletion of this nutrient considerably affects the photosynthetic process (Suganya et al. 2016). Microalgae perform active transportation at the plasma membrane for the uptake of orthophosphorus in the forms of $H_2PO_4^-$ and HPO_4^{2-}. During algal metabolism, PO_4^{3-}-P is merged into the organic compounds by the following mechanism: (i) oxidative phosphorylation, (ii) substrate-level phosphorylation and (iii) photophosphorylation. Mainly these mechanisms include the production of ATP from ADP and energy input. In the first operation, energy is grabbed from the ETS (electron transport system) occurring in mitochondria, and in the second operation, respiratory substrate is oxidized to provide energy input. In the third process, it is obtained from the transformation of the light energy. The general reaction of phosphorylation has been represented in Eq. (16.3) (Martinez Sancho et al. 1997):

$$\mathrm{ADP} + \mathrm{P_i} \xrightarrow{\text{Energy}} \mathrm{ATP} \tag{16.3}$$

When there is a shortage of inorganic phosphate, microalgal cells convert organic phosphate to orthophosphate by phosphatase present on the surface of the cell and utilize them. When there is an excess of phosphate, microalgal cells assimilate and store them within the cells in the form of polyphosphate (volatin) granules. These granules are then utilized for continued growth during the shortage of phosphate in the growth medium/environment (Kuenzler 1965; Larsdotter 2006; Whitton and Potts 2007).

16.3.4 Parameters Influencing Nutrient Removal by Microalgae

Environmental conditions of wastewater such as pH, dissolved oxygen, temperature and light are the crucial parameters that affect the nutrient uptake by microalgal cells. Carbon assimilation mechanism performed by microalgal cells strongly depends upon the pH, as this factor regulates the solubility of CO_2 in the medium. The high pH of the medium is responsible for the PO_4^{3-}-P precipitation and stripping of NH_4^+-N. Dissolved oxygen concentration also affects the removal of PO_4^{3-}-P and NH_4^+-N. Regarding the temperature effect, it is reported that when the

temperature of the medium reaches the optimal values, it results in higher reaction rates of metabolic activities and hence high nutrient uptake rate. Also, as the temperature increases to a higher value, it results in the decreased solubility of nutrients such as NH_4^+-N and CO_2. Generally, for the autotrophic mode of microalgae cultivation, increased light intensity and duration of supply results in higher RE (Goncalves et al. 2017).

16.4 Remediation of Heavy Metals

Microalgae possess the ability to uptake heavy metals (HMs) from wastewater. Thus, the concentration of HMs in microalgal cells is higher in comparison with the surrounding medium (Megharaj et al. 2003; Priyadarshani et al. 2011). Microalgae perform various HM sorption processes involving different metabolisms (Ajayan et al. 2011). The uptake process generally includes two steps: (i) initially, metals are rapidly sorbed at the cell surface, and (ii) detoxification of HMs by slower metabolic process occurs within the cell. Advantages of using microalgae for metal bioremediation process include (i) capability of metal uptake at faster rate in comparison with other adsorption techniques; (ii) time and energy saving; (iii) faster growth rates; (iv) can bind up to 10% of their biomass; (iv) application in both batch and continuous process; (v) eco-friendly, recyclable or reusable; and (vi) applicability in wastewater treatment (Monteiro et al. 2012).

Dirbaz and Roosta (2018) examined four microalgae species, namely, *Spirulina* sp., *Parachlorella* sp., *Nannochloropsis* sp. and *Scenedesmus* sp., for the biosorption capability of Cd^{2+} ions from aqueous solution. *Parachlorella* sp. showed the highest biosorption capacity which was 90.72 mg/g (mass of sorbate/mass of sorbent) at 30 °C and pH 7. It was 1.5–3 times greater than other biosorbents investigated. Biosorption by *Parachlorella* was further optimized, and a maximum uptake of 96.20 mg g^{-1} at 35 °C and pH 7 was reported. Effect of agitation rate was also studied. When the agitation rate was increased to 250 rpm or higher, the uptake of heavy metals was reduced to less than half of the initial bioaccumulation rate (Dirbaz and Roosta 2018). In another study, microalgae species (*Chlorella vulgaris*, *Chlamydomonas reinhardtii*, *Scenedesmus almeriensis* and a native *Chlorophyceae* sp. isolated from Loa River, Spain) were compared for the uptake of arsenic (As), zinc (Zn), manganese (Mn), copper (Cu) and boron (B) in both monometallic and multimetallic solution, and the effects of contact time and pH on sorption were evaluated. The maximum RE for each metal ion that was obtained by different microalgae species is presented in Table 16.2 (Saavedra et al. 2018).

Immobilization techniques are also used to enhance the sorption capacity of microalgal biomass. Ahmad et al. (2018) investigated the use of both free and immobilized *C. vulgaris* biomass for the sorption of ferrous (Fe^{2+}), manganese (Mn^{2+}) and zinc (Zn^{2+}) ions. *C. vulgaris* biomass was trapped in calcium alginate beads. The authors also studied the effects of initial metal-ion concentration, contact time, biosorbent dosage and pH. Immobilized biomass achieved the maximum biosorption which was 129.83 mg/g for Fe^{2+}, 115.90 for Mn^{2+} and 105.29 for Zn^{2+} at

Table 16.2 Removal efficiency obtained for each metal ion (Saavedra et al. 2018)

S. No.	Metal ion	Microalgae species	pH	Contact time	Removal efficiency (RE)
1	Mn	*C. vulgaris*	7.0	3 h	99.4%
2	Zn	*Chlorophyceae* sp.	5.5	3 h	91.9%
3	Cu	*Chlorophyceae* sp.	7	10 min	88%
4	B	*S. almeriensis*	5.5	10 min	38.6%
5	As	*S. almeriensis*	9.5	3 h	40.7%

optimal pH of 6.0 and biosorbent dosage of 0.4 g/L with contact time of 5 h at 25 °C (Ahmad et al. 2018). Table 16.3 presents the heavy metal bioremediation capability of various microalgal species.

Table 16.3 shows that *Chlorella*, *Scenedesmus* and *Spirulina* species are most widely applied for metal-ion uptake from the liquid phase. Also, metal-ion uptake capability is affected by pH of the medium. Effect of pH on metal-ion sorption by microalgal cells has been discussed later in this review.

16.4.1 Interaction of HMs with the Cell Wall of Microalgae

The interface of the HMs at the cell wall of microalgae have been proposed as the initial step of metal sorption as in initial condition; metal cation translocates through the microalgal cell wall. Metallic species react with carbohydrates, lipid and protein present on the external surfaces of the cell wall (Crist et al. 1981; Cetinkaya Donmez et al. 1999; Monteiro et al. 2012). During the interaction, HMs form a distinctive coordination complex with the functional groups such as sulphate, carboxyl, and amino group of protein and polysaccharide, imidazole of histidine and nitrogen and oxygen of peptide bond. Such type of complex formation occurs by proton displacement, and the pH of the medium is a crucial parameter in determining the extent of protonation. Some unprotonated carboxyl oxygen and sulphate also get electrostatically bonded to HMs (Crist et al. 1981). Figure 16.4 (Part I) presents the As uptake by microalgal cells through ion exchange mechanism.

16.4.2 Interaction with the Plasma Membrane

The transport and detoxification of HMs (Fig. 16.4; Part II) through plasma membrane are crucial processes. Microalgal cells interact with the external environment through metal transporters. Generally, the transporters are placed into two groups: Group A and Group B (Blaby-Haas and Merchant 2012). Metal ions are translocated into the cytoplasm through the membrane by Group A transporters. Members of Cu transporter (CTR), natural resistance-associated macrophage protein (NRAMP), Fe transporter (FTR) and Zrt- and Irt-like protein (ZIP) families

Table 16.3 Metal uptake by various species of microalgae

Metallic species	Microalgal species	Initial concentration of metal	Temp. (°C)	Medium pH	Amount of metal uptake (mg/g)	Reference
Cd^{2+}	*Chaetoceros calcitrans*	1 ppm	20–22	8	1055.27	Sjahrul and Arifin (2012)
	Desmodesmus pleimorphus (ACOI 561)	5.0 mg L^{-1}	25	4	85.3	Monteiro et al. (2010)
	Desmodesmus pleimorphus (L)	5.0 mg L^{-1}	25	4	61.2	Monteiro et al. (2010)
	Planothidium lanceolatum	100 mg L^{-1}	25	7	275.51	Sbihi et al. (2012)
	Tetraselmis chuii	1 ppm	20–22	8	13.46	Sjahrul and Arifin (2012)
	Scenedesmus abundans	1 mg L^{-1}	25	7.8–8	11.5	Monteiro et al. (2009)
	Ulva prolifera	100 μM	20	Na	100.633 ± 15.711 μg/g	Huan et al. (2018)
Co	*Chlamydomonas reinhardtii*	15 μM L^{-1}	25	5.5	0.89	Macfie and Welbourn (2000)
	Chlamydomonas reinhardtii (without cell wall)	15 μM L^{-1}	25	5.5	1.3	Macfie and Welbourn (2000)
Cr^{3+}	*Spirulina* sp.	0.05–0.5 g/25 ml	Na	Na	304	Doshi et al. (2007)
	Spirulina sp. (HD-104)	Na	Na	Na	306	Doshi et al. (2008)
	Scenedesmus quadricauda	100 mg L^{-1}	22.3	6	58.47	Shokri Khoubestani et al. (2015)
Cr^{+6}	*Chlamydomonas reinhardtii*	1000 mg/L	22	2	25.6	Arica et al. (2005)
	Spirulina sp.	0.05–0.5 g/25 ml	Na	Na	333	Doshi et al. (2007)
$Cr_2O_7^{-2}$	*Spirulina* sp. (HD-104)	Na	Na	Na	226	Doshi et al. (2008)

(continued)

Table 16.3 (continued)

Metallic species	Microalgal species	Initial concentration of metal	Temp. (°C)	Medium pH	Amount of metal uptake (mg/g)	Reference
Cu^{2+}	*Anabaena cylindrica*	450 μg L^{-1}	23	4.0–5.0	12.62	Tien et al. (2005)
	Asterionella formosa	450 μg/L	23	4.0–5.0	1.1	Tien et al. (2005)
	Chlorella vulgaris	5 mg/L	24 ± 2	4.5	63.08	Mehta and Gaur (2001)
	Planothidium lanceolatum	100 mg L^{-1}	25	7	134.42	Sbihi et al. (2012)
	Spirulina platensis	2.55–3.8 mg L^{-1}	34	9	0.85	Nalimova et al. (2005)
	Spirulina sp. (HD-104)	Na	Na	Na	576	Doshi et al. (2008)
Fe^{3+}	*Chlorella vulgaris*	Na	Na	2	24.52	Romera et al. (2006)
	Microcystis sp.	50 μg ml^{-1}	29 ± 2	9.2	0.03	Singh et al. (1998)
Hg^{2+}	*Chlamydomonas reinhardtii*	25–500 mg/L	25	6	106.6	Bayramoglu et al. (2006)
	Pseudochlorococcum typicum	0–100 μg/L	20 ± 1	7	15.13	Shanab et al. (2012)
Ni^{2+}	*Chlorella miniata*	10–40 μg/ml	24 ± 1	7.4	1.37	Wong et al. (2000)
	Chlorella vulgaris	1000 ppm	25	5	15.4	Abu Al-Rub et al. (2004)
	Arthrospira platensis	0.5–3.0 mM	30 ± 1	5.0–5.5	20.78	Ferreira et al. (2011)
	Spirulina	0.05–0.5 g/25 ml	Na	Na	1378	Doshi et al. (2007)
Pb^{2+}	*Chlamydomonas reinhardtii*	25–500 mg/L	25	6	380.7	Bayramoğlu et al. (2006)
	Oscillatoria laete-virens	10–100 mg/L	25 ± 2	5	21.6	Miranda et al. (2012)
	Pseudochlorococcum typicum	0–100 μg L^{-1}	20 ± 1	7	4.49	Shanab et al. (2012)
	Spirulina platensis	5–100 μg L^{-1}	25 ± 1	7	188	Arunakumara et al. (2008)
Zn^{2+}	*Cyclotella cryptica*	0.5 mg L^{-1}	25 ± 2	6	242.9	Schmitt et al. (2001)
	Planothidium lanceolatum	100 mg/L	25	7	118.66	Sbihi et al. (2012)
	Scenedesmus subspicatus	0.5 mg/L	20 ± 2	6	72.06	Schmitt et al. (2001)

constitute Group A transporters. This group also includes the assimilative transporters present in the membrane and increases the concentration of HMs in the cytosol. The membrane of the vacuole also has Group A transporters which perform similar function as assimilative transporters, but they uptake metal ions present within the cell. The function of Group B transporters is to decrease the concentration of metal ions present in the cytoplasm such as providing metal ions for binding to the metal-dependent proteins localized in the organelles of the cell. This group consists of members from the P1B-type ATPase, ferroportin (FPN), cation diffusion facilitator (CDF) and Ca(II)-sensitive cross-complementer 1 (Ccc1)/vacuolar iron transporter 1 (VIT 1) families (Blaby-Haas and Merchant 2012).

16.4.3 Physical Adsorption

The physical adsorption process is not dependent upon the metabolism of the microalgal cell, and also it is a reversible process offering several advantages. In this process, polyelectrolytes present on the cell wall bind to the metal ions through electrostatic interaction (van der Waals forces, redox interactions, covalent bonding and biomineralization), and thus they achieve electroneutrality (Perpetuo et al. 2011). Ionic interactions occurring between the cell walls and the metal ions are responsible for biosorption of cadmium, uranium and zinc (Kuyucak and Volesky 1988). In the same way, copper is physically adsorbed by alga *C. vulgaris* through van der Waals forces (Aksu et al. 1992).

16.4.4 Role of Microalgal Organic Acids

The microalgal organic acids (e.g. lactic, citric, fumaric, oxalic, malic and gluconic) perform two functions: (i) formation of metalloorganic molecules by chelating toxic metal ions and (ii) leaching and solubilization of metal components from the cell surfaces (Perpetuo et al. 2011).

16.4.5 Precipitation

When the pH of the solution is low, functional sites of the cell wall are blocked by protons. Thus metal ions are restricted from binding due to repulsive forces. As the medium pH increases, the protons are displaced by negative charges from functional sites. This results to an increase in adsorption of HMs on the functional sites. The decrease in the solubility of metallic ions results in the reduction of their bioavailability, and precipitation of ions takes place subsequently (Perpetuo et al. 2011).

Cellular metabolism plays a crucial role in precipitation process, as it may either depend on or may be independent of the cellular metabolism: (i) When the precipitation depends upon the metabolism, microbes secrete specific compounds that

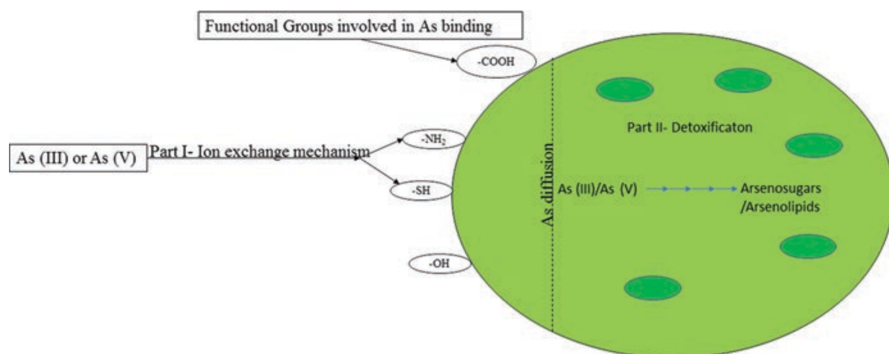


Fig. 16.4 Flow diagram of As ion biosorption (Part I) and detoxification of As ions (Part II) by microalgae

cause precipitation when they encounter a metal ion; and (ii) when the precipitation process is independent of cellular metabolism, precipitation may be due to interaction between the cell surface and metal, or another biosorption process may take place simultaneously (Ahayla and Ramachandra 1995). It was reported that precipitation of Cd^{2+}occurred in the vacuole of *Tetraselmis suecica* (Ballan-Dufrancais et al. 1991).

16.4.6 Metallothioneins

Valle and coworkers first characterized metallothioneins in the late 1950s (Stillman 1995). Metallothioneins are proteinaceous in nature, generally low molecular weight (approx. 6–7 kDa), structurally diverse and cysteine-rich and form complex with HMs in thiol cluster. These peptides are grouped into two categories: (i) phytochelatins (Class III metallothioneins or MtIII) are synthesized by enzymes in the form of short-chain polypeptides and found in certain fungi, algae and higher plants; (ii) MtII (Class II metallothioneins – reported in algae, cyanobacteria and higher plants), and MtI (Class I metallothioneins) and observed in *Neurospora* and *Agaricusbisporus* (not identified in algae) found in most vertebrates, both are encoded by genes (Perales-Vela et al. 2006). Several investigations have been performed by researchers that confirmed Class III metallothioneins are synthesized and present in algae (Gekeler et al. 1989; Robinson 1989a, b; Gaur and Rai 2001). Also, in vitro studies reported that a stable complex is formed when HMs bind to long-chain MtIII (Mehra et al. 1995; Perales-Vela et al. 2006). These molecules reduce the cytosolic free metal-ion concentration by chelating them such as Cd and other metallothioneins believed to perform Zn and Cu homeostasis (Robinson 1989a, b). Several HMs such as Ni^{2+}, Cd^{2+}, As^{3+}, Ag^{+}, Pb^{2+}, Bi^{3+}, Hg^{2+}, Cu^{2+}, Cu^{2+}, Au^{2+} and Zn^{2+} induce the synthesis of MtIII both in vivo and in vitro (Robinson 1989a, b; Ahner and Morel 1995; Knauer et al. 1997; Pawlik-Skowronska 2003a, b; Pawlik-Skowronska et al. 2004; Perales-Vela et al. 2006).

16.4.7 Role of the Vacuole in Metal Sequestration

Chlorella salina was investigated to study the RE of three metal ions (Co, Zn and Mn), and it was detected that higher concentration of HMs was present in the vacuole instead of the cytosol (Garnham et al. 1992). The mechanism of this phenomenon could be (i) HM regulation within the cytoplasm or (ii) metal-ion detoxification (Monteiro et al. 2012). Microscopical and X-ray studies showed that metal ion was complexed with MtIII and then transported into the vacuoles of microalgal cells (Perales-Vela et al. 2006). Few electron-dense materials made up of cadmium and sulphur (in ratios between 2 and 2.4) were observed in the vacuoles of the microalga *Dunaliella bioculata* (Heuillet et al. 1986). Other studies also reported the presence of Cd^{2+} in the vacuoles of green alga *Tetraselmis suecica* (Ballan-Dufrançais et al. 1991) and diatom *Skeletonema costatum* (with the presence of Cu^{2+} too) having sulphur-to-metal ratio of 1.5 (Nassiri et al. 1997). Dark and spherical electron bodies detected in the vacuoles of the three freshwater microalgal cells {*Scenedesmus quadricauda* var. *quadrispina*, *Pseudochlorococcum typicum* (Chlorophyta) and *Phormidium ambiguum* (*Cyanobacteria*)} were exposed to Pb^{2+} ions (Shanab et al. 2012). In this phenomenon, metal ions either bind or form complexes with phytochelatin or form metallo-iron, metallo-sulphur or metallo-phosphate complex and then transport from cytosol to vacuole. In the vacuole, high concentration of organic acid is present, which releases the metal from complex and returns the peptide to the cytosol (Shanab et al. 2012).

16.4.8 Role of the Chloroplast and Mitochondria

When microalgal species {*Scenedesmus quadricauda* var. *quadrispina*, *Pseudochlorococcum typicum* (Chlorophyta) and *Phormidium ambiguum* (*Cyanobacteria*)} isolated from fresh water were exposed to Hg^{2+}, Pb^{+2} and Cd^{2+}, it was observed that excessive starch was accumulated in the chloroplast (around the pyrenoids) in aqueous solution (Shanab et al. 2012). This study showed the possibility of accumulation of heavy metals in other organelles such as mitochondria. In another study, the accumulation of Cd^{2+} inside the chloroplast was also observed in *Chlamydomonas reinhardtii* (Nagel et al. 1996). Sequestration of Cd^{2+} in mitochondria and chloroplast may occur due to any of the following processes: (i) Complex formation of MtIII with Cd^{2+} in the cytosol and then transfer of these complexes into the mitochondria and chloroplast; (ii) Cd^{2+} binds to the MtIII synthesized inside the organelle, which translocated as free ions, and then forms high molecular weight (HMW) compounds; or (iii) the above two processes occur at the same time, and MtIII are synthesized in all the three cellular compartments (chloroplast, mitochondria and cytosol) (Mendoza-Cozatl et al. 2005; Perales-Vela et al. 2006).

When *Oocystis nephrocytioides* was grown in medium containing Cu^{2+}, a high concentration of Cu^{2+} was accumulated in the pyrenoids and thylakoids. Localization of Cu^{2+} suggests that its interaction with the ligands is confined in the chloroplast

(Soldo et al. 2005). On the other way, transportation of Cu^{2+} from the cytosol to the chloroplast can occur by the formation of Cu^{2+}-ligand complex (Perales-Vela et al. 2006).

16.5 Kinetics of Substrate Removal

In order to operate large-scale microalgae production system for the long term, it is required to supply essential nutrients (N, C, P and trace elements) for effective growth. Therefore, it is required to determine the nutrient removal kinetic models for the continuous large-scale production of microalgae. Various kinetics models which have been applied to determine the suitable substrate removal kinetics are as follows.

16.5.1 Michaelis-Menton Kinetics

Michaelis-Menten kinetics is used to determine the batch kinetic coefficients represented by Eq. (16.4) (Aslan and Kapdan 2006):

$$R = \frac{R_{\max} S}{K_m + S} \tag{16.4}$$

where R is the rate of substrate removal, S is the concentration of substrate in the effluent, R_{max} is the maximum rate of substrate removal and K_m is constant indicating saturation (or half saturation constant). The initial concentration of substrate (S_o) and the initial rate of substrate assimilation (R_{so}) are considered in a batch operation. On modification, Eq. (16.4) becomes:

$$R_{so} = \frac{R_{mo} S_o}{K_m + S_o} \tag{16.5}$$

where $R_{mo}$is the initial maximum substrate removal rate given by $R_{mo}= k.X_o$. Now, Eq. (16.5) can be written as:

$$R_{so} = \frac{k X_o S_o}{K_m + S_o} \tag{16.6}$$

where k indicates the reaction rate constant (time^{-1}) and X_o corresponds to the initial concentration of biomass. The specific substrate removal rate (R_{Xi}) is calculated by dividing R_{so} to X_o:

$$R_{Xi} = \frac{R_{so}}{X_o} = \frac{k S_o}{K_m + S_o} \tag{16.7}$$

Linearized form of Eq. (16.7) has been shown in Eq. (16.8):

$$\frac{1}{R_{Xi}} = \frac{1}{k} + \frac{K_m}{k} \frac{1}{S_o} \tag{16.8}$$

On plotting $1/R_{Xi}$ and $1/S_o$, it generates a straight line curve with a slope of K_m/k and an intercept of $1/k$.

Yield coefficients for the P and N removal are determined by Eqs. (16.9) and (16.10), respectively (Aslan and Kapdan 2006; Wang et al. 2014):

$$(chl\,a)_f - (chl\,a)_i = Y_P\left[(P)_0 - (P)_f\right] \tag{16.9}$$

$$(chl\,a)_f - (chl\,a)_i = Y_N\left[(N)_0 - (N)_f\right] \tag{16.10}$$

where $(chl\ a)_f$ and $(chl\ a)_i$ correspond to the final and the initial concentration of chl a (mg/L) and Y_N and Y_P are the yield coefficients for N and P, respectively. N_0 and N_f are the initial and final concentrations of N (mg/L), respectively, and P_0 and P_f are the initial and final concentrations of P (mg/L), respectively. Table 16.4 presents the kinetic coefficients calculated by using Michaelis-Menten kinetics using different microalgal species and wastewater sources.

It can be concluded from Table 16.4 that the removal rate of ammonia is higher than that of phosphorus as in case of ammonia reaction rate constant is higher than that for phosphorus.

16.5.2 Logistic Model and Luedeking-Piret Model

The logistic model is used for determining the biomass productivity and microalgal growth rate, which is given by Eq. (16.11); an integral expression of this equation is represented by Eq. (16.12) (Ruiz et al. 2013a, b):

$$\frac{dX}{dt} = \mu X\left(1 - \frac{X}{X_m}\right) \tag{16.11}$$

$$X(t) = \frac{X_0 X_m e^{\mu_m t}}{X_m - X_0 + X_0 e^{\mu_m t}} \tag{16.12}$$

where X represents the concentration of biomass (mg/L) at any time, t, X_o is the initial concentration of biomass (mg/L), μ_m is the maximum growth rate (d^{-1}), μ represents the specific growth rate (d^{-1}) and X_m is the maximum concentration of biomass (mg/L).

Luedeking-Piret model has been applied for the determination of substrate consumption rate expressed as:

$$\frac{dS}{dt} = -\frac{1}{Y_x}\left(\frac{dX}{dt}\right) - mX \tag{16.13}$$

On integrating the above equation and using the logistic equation, Eq. (16.13) becomes:

Table 16.4 Kinetic coefficients determined for different microalgal species based on Michaelis-Menten kinetics

Microalgal species	Wastewater source	Component	Kinetic coefficients			Reference
			k	K_m	Y	
Chlorella vulgaris	Synthetic wastewater	N(NH_4-N)	1.5 mg NH_4^+-N/mg chl a/d	31.5 mg/L	0.15 mg chl a/mgNH_4^+-N	Aslan and Kapdan (2006)
		P(PO_4-P)	0.5 mg PO_4^{3-}-P/mg chl a/d	10.5 mg/L	0.14 mg chl a/mgPO_4^{3-}-P	
Chlorella sp.	Primary effluent	N			0.18 mg chl a/mgN	Wang et al. (2014)
		P		3.01 mg/L	1.6 mg chl a/mgP	
	Mixture of primary effluent and anaerobic digestion centrate	N			0.1	
		P		3.01 mg l^{-1}	2.4 mg chl a/mg^{-1}P	
Micractinium sp.	Primary effluent	N			0.23 mg chl a/mg^{-1}N	Wang et al. (2014)
		P		4.2 mg l^{-1}	2 mg chl a/mg^{-1}P	
	Mixture of primary effluent and anaerobic digestion centrate	N			0.12 mg chl a/mg^{-1}N	
		P		4.2 mg l^{-1}	2.9 mg chl a/mg^{-1}P	

(continued)

Table 16.4 (continued)

Microalgal species	Wastewater source	Component	Kinetic coefficients			Reference
			k	K_m	Y	
Chlorella sorokiniana	Palm oil mill effluent	Nitrate	$9.2 * 10^{-3}$ mg NO_3^- mg^{-1}DCW d^{-1}	68.7 mg/L	0.1 g DCW g^{-1} NO_3^-	Saidu et al. (2017)
		Phosphate	$8 * 10^{-3}$ mg PO_4^{3-} mg^{-1} DCW d-1	144.6 mg/L	0.12 g DCW g^{-1} PO_4^{3-}	
		Ammonium	$2.3 * 10^{-2}$ mg NH_4^+ mg^{-1}DCW d^{-1}	113 mg/L	0.08 g DCW g^{-1} NH_4^+	
		COD	0.15 mg COD mg^{-1}DCW d^{-1}	1662 mg/L	0.02 g DCW g^{-1} COD	
Gracilaria foliifera	Seawater	Ammonium		0.2 ± 0.1 μM		Deboer et al. (1978)
		Nitrate		0.4 ± 0.1 μM		
Neoagardhiella baileyi	Seawater	Ammonium		0.2 ± 0.1 μM		Deboer et al. (1978)
		Nitrate		0.2 ± 0.2 μM		
Prorocentrum donghaiense	Artificial seawater (nitrate deplete)	Nitrate		1.3 ± 0.1 μmol N L^{-1}		Hu et al. (2014)
		Ammonium		5.3 ± 1.1 μmol N L^{-1}		
		Urea		0.13 ± 0.01 μmol N L^{-1}		
		Algal amino acids		9.9 ± 0.9 μmol N L^{-1}		
	Artificial seawater (NO_3^{-2} replete cultures (50 NO_3^{-2})	Ammonium		7.1 ± 0.4 μmol N L^{-1}		
		Urea		0.12 ± 0.01 μmol N L^{-1}		
		Algal amino acids		12.5 ± 0.1 μmol N L^{-1}		

Chlorella vulgaris	SMF (sterilized manure feedstock)					Pandey (2017)
	9 days cultivation	Nitrogen	0.39 mg TN/mg biomass day	60.7 mg/L	25.5 mg biomass/mg TN	
		Phosphorus	2.0 mg TP/mg biomass day	190.8 mg/L	31.5 mg biomass/mg TP	
	30 days cultivation	Nitrogen	0.44 mg TN/mg biomass day	69.5 mg/L	140.1 mg biomass/mg TN	
		Phosphorus	0.92 mg TP/mg biomass day	0.25 mg/L	108.3 mg biomass/mg TP	
	MFM (untreated manure feedstock media)					
	9 days cultivation	Nitrogen	6.29 mg TN/mg biomass day	278.6 mg/L	31.5 mg biomass/mg TN	
		Phosphorus	102.0 mg TP/mg biomass day	0.40 mg/L	114.5 mg biomass/mg TP	
	30 days cultivation	Nitrogen	44.6 mg TN/mg biomass day	1538.9 mg/L	64.7 mg biomass/mg TN	
		Phosphorus	106.4 mg TP/mg biomass day	0.09 mg/L	78.7 mg biomass/mg TP	
Coimmobilized microalgae and nitrifying bacteria	Synthetic wastewater	Ammonium		4.9 g/L		Bai et al. (2016)
Catenella nipae	Artificial seawater	Ammonium		692 µM		Runcie et al. (2003)
		Nitrate		5 µM		
Ulva lactuca	Artificial seawater	Ammonium		85 µM		Runcie et al. (2003)
		Nitrate		34 µM		

$$S(t) = S_0 - \frac{1}{Y_x}\frac{X_0 X_m e^{\mu_m t}}{X_m - X_0 + X_0 e^{\mu_m t}} - m\frac{X_m}{\mu_m}\ln\left(\frac{X_m - X_0 + X_0 e^{\mu_m t}}{X_m}\right) \quad (16.14)$$

where S is the rate-limiting substrate concentration at time t (mg/L), S_o is the initial concentration of substrate (mg/L) that may be rate limiting, Y_x is the observed biomass yield coefficient (mg/mg) and m corresponds to cell maintenance coefficient (d^{-1}). Table 16.5 represents the substrate removal parameters determined through Luedeking-Piret model.

16.5.3 Stover-Kincannon Kinetics

During continuous operation of the photobioreactor, Stover-Kincannon equation provides a suitable way for determining the substrate removal rate represented by Eq. (16.15) (Karapinar Kapdan and Aslan 2008):

$$S_{e=}S_o - \frac{U_{\max} S_o}{K_B + (QS_o / V)} \quad (16.15)$$

where S_e (mg/L) indicates the concentration of effluent substrate, S_o (mg/L) indicates the concentration of influent substrate, V is the reactor liquid volume (L), Q corresponds to flowrate (L/day), U_{max} indicates the maximum rate of substrate removal and K_B is the saturation constant. In the study of ammonium-nitrogen assimilation by *C. vulgaris* cultivated in a continuously operated photobioreactor, K_B and U_{max} were determined as 10.3 and 13.0 mg/L/day (Karapinar Kapdan and Aslan 2008).

16.5.4 Gompertz Model

This model was simplified to determine nutrient removal kinetics for microalgal cells and represented by Eq. (16.16) (Goncalves et al. 2016):

$$S(t) = S_i + (S_f - S_i) \times \exp(-\exp\left[k \times (\lambda - t) + 1\right] \quad (16.16)$$

where $S(t)$ indicates the concentration of substrate at time t; S_i and S_f are the initial and final concentration of substrate (mgL^{-1}), respectively; k corresponds to rate of substrate uptake (d^{-1}); and λ corresponds to lag time (d). The biomass yield ($Y_{X/S}$) and substrate RE (*%R*) were calculated according to Eqs. (16.17) and (16.18), respectively (Goncalves et al. 2016):

$$Y_{X/S} = \frac{X_f - X_i}{S_i - S_f} \quad (16.17)$$

Table 16.5 Parameters for substrate removal determined using Luedeking-Piret model

Microalgal species	Wastewater source	Element	Parameters		Reference
			$1/Y_x$	m	
Chlorella vulgaris CEW-1	Cellulosic ethanol wastewater				Li et al. (2017)
	PBR a	COD	316.02 mg/mg	7.754 d^{-1}	
		TAN	162.59 mg/mg	3.762 d^{-1}	
		TP	20.98 mg/mg	0.510 d^{-1}	
		Total	499.59 mg/mg	12.026 d^{-1}	
	PBR b	COD	303.47 mg/mg	8.246 d^{-1}	
		TAN	161.59 mg/mg	4.540 d^{-1}	
		TP	20.96 mg/mg	0.551 d^{-1}	
		Total	486.00 mg/mg	13.337 d^{-1}	
	PBR c	COD	235.06 mg/mg	13.354 d^{-1}	
		TAN	132.62 mg/mg	7.912 d^{-1}	
		TP	16.78 mg/mg	0.866 d^{-1}	
		Total	384.46 mg/mg	22.132 d^{-1}	
Chlorella minutissima UTEX2341		Glycerine (9.02 g/L)	0.8629 g g^{-1}	0.0001 g $g^{-1}d^{-1}$	Yang et al. (2011)
		(14.5 g/L)	0.8374 g g^{-1}	0.001 g $g^{-1}d^{-1}$	
		(25.2 g/L)	0.8543 g g^{-1}	3.11E-07 g $g^{-1}d^{-1}$	
Chlorella pyrenoidosa	Medium containing glucose and treated molasses	Glucose (10 g/L)	0.04 g/g	0.863 g $g^{-1}d^{-1}$	Gaurav et al. (2016)
		Treated molasses (10 g/L)	0.836 g/g	0.75 g $g^{-1}d^{-1}$	
Isochrysis galbana	Seawater	Sodium nitrate			He et al. (2016)
		(25 mg/L)	0.065 ± 1.1 mg/mg	$5.5 \times 10^{-4} \pm 3.4 \times 10^{-4}$ mg mg^{-1}	
		(50 mg/L)	0.073 ± 0.74 mg/mg	$3.2 \times 10^{-4} \pm 8.1 \times 10^{-4}$ mg mg^{-1}	
		(75 mg/L)	0.142 ± 2.4 mg/mg	$-3.14 \times 10^{-3} \pm 1.04 \times 10^{-3}$ mg mg^{-1}	
		(100 mg/L)	0.144 ± 1.8 mg/mg	$-3.28 \times 10^{-3} \pm 1.62 \times 10^{-3}$ mg mg^{-1}	

Table 16.5 (continued)

Microalgal species	Wastewater source	Element	Parameters		Reference
			$1/Y_x$	m	
Chlorella vulgaris	Urban wastewater	Ammonium			Taylor et al. (2011)
		(5.8 mg/L)	0.086 g/g	Na	
		(11.8 mg/L)	0.080 g/g	Na	
		(19.7 mg/L)	0.066 g/g	Na	
		(23.9 mg/L)	0.020 g/g	Na	
		(49.mg/L)	0.024 g/g	Na	
		(117.5 mg/L)	0.071 g/g	Na	
		(226.8 mg/L)	0.045 g/g	Na	
		Phosphate			
		(1.3 mg/L)	2.173 g/g	Na	
		(2.0 mg/L)	2.767×10^{-3} g/g	Na	
		(5.3 mg/L)	3.501×10^{-3} g/g	Na	
		(9.7 mg/L)	2.125×10^{-3} g/g	Na	
		(25.3 mg/L)	3.745×10^{-3} g/g	Na	
		(51.4 mg/L)	0.214 g/g	Na	
		(143.5 mg/L)	25 g/g	Na	

Table 16.6 Kinetic constants of nutrient assimilation determined through Gompertz model (Goncalves et al. 2016)

Microalgal species	Wastewater source	Component	$Y_{x/s}$(g_{dw} g^{-1})	λ (d)	k (d^{-1})
C. vulgaris		N	2.1	0.31	0.63
S. salina + *C. vulgaris*			2.8	0.35	0.50
M. aeruginosa		P	9.5	0.73	0.54
S. salina + *M. aeruginosa*			12.9	1.57	0.68

$$\%R = \frac{S_i - S_f}{S_i} \times 100 \quad (16.18)$$

Goncalves et al. (2016) performed a study for the determination of removal efficiency of nutrients by two microalgal species *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* and two cyanobacterial species *Microcystis aeruginosa* and *Synechocystis salina* cultivated both in single-culture mode and co-culture mode containing *S. salina* (*P subcapitata* + *S. salina*, *C. vulgaris* + *S. salina*, *M. aeruginosa* + *S. salina*). Table 16.6 presents the highest nitrogen and phosphorus RE which was obtained in terms of biomass yield ($Y_{x/s}$) and kinetic constants of substrate removal determined through Gompertz model (λ and k) (Goncalves et al. 2016).

Table 16.6 clearly shows that the co-culture cultivation mode has a higher rate of removal efficiency.

16.5.5 Biomass-Dependent Growth Model

In order to describe removal kinetics, two expressions were chosen to determine the removal rate with [Eq. (16.19)] and without [Eq. (16.20)] dependence of biomass (Ruiz et al. 2013a, b):

$$-\frac{dS_a}{dt} = k \cdot S_a \cdot X \quad (16.19)$$

$$-\frac{dS_a}{dt} = k \cdot S_a \quad (16.20)$$

where k corresponds to kinetic constant, S_a is the concentration of assimilable substrate and X is the concentration of biomass at time t. At the time of cultivation period, total substrate concentration (S) is used for experimental analysis. Therefore, performing the mathematical conversion of the above two equations results in two models: (i) Model 1, represented by Eq. (16.21), and (ii) Model 2, expressed by Eq. (16.22) describing the substrate variation pattern as given below (Murwanashyaka et al. 2017):

Table 16.7 Maximum kinetic parameters for nutrient removal determined using Model 1 and Model 2 (Murwanashyaka et al. 2017)

Condition	For nitrogen removal		For phosphorus removal	
Kinetic parameters	p(day^{-1})	k(day^{-1})	p(day^{-1})	k(day^{-1})
Varying initial nitrogen concentration	4.47	1.11	4.68	1.63
Varying initial nitrogen concentration	4.98	1.38	6.90	3.34

p and *k* stand for maximum removal rate and kinetic constant, respectively

$$S = \frac{\left(\frac{X_o}{Y} + S_o\right)\left(S_o - S_{na}\right) + \frac{X_o}{Y} S_{na} \exp\left(pt\right)}{\left(S_o - S_{na}\right) + \frac{X_o}{Y} \exp\left(pt\right)} \tag{16.21}$$

$$S = S_{na} + \left(S_o - S_{na}\right) \exp\left(-k \bullet t\right) \tag{16.22}$$

where S_o and S_{na} are the initial and non-assimilated concentration of substrate, respectively (g L^{-1}), *Xo* is the initial concentration of biomass (g L^{-1}), *p* corresponds to the maximum specific removal rate of substrate (d^{-1}) and *Y* corresponds to the yield coefficient of biomass (g g^{-1}) calculated by Eq. (16.23) (Murwanashyaka et al. 2017):

$$Y = \frac{X - X_o}{S_o - S_{na}} \tag{16.23}$$

Murwanashyaka et al. (2017) performed a study in order to investigate the capability of *Chlorella sorokiniana* FACHB-275 to treat wastewater by varying the initial concentration of nitrogen keeping initial phosphorus concentration constant or vice versa. Table 16.7 presents the maximum removal rate (*p*) for nitrogen and phosphorus that was obtained in the above study.

16.6 Conclusion

The investigation concludes that microalgae possess the ability to remediate nutrients from wastewater with removal efficiency of more than 90% with the simultaneous recovery of nutrients. *Chlorella* and *Scenedesmus* spp. are most widely studied and exploited for the wastewater treatment. Understanding the mechanism of nutrient uptake in more detail will help to maximize the nutrient uptake process. Microalgal cells also show high capability for accumulation, adsorption and recovery of heavy metals. Thus microalgae represent the inexpensive route for the development of promising biosorbents. Stover-Kincannon kinetics and Michaelis-Menton kinetics are widely used for determination of kinetic coefficients for large-scale wastewater treatment and microalgae biomass production.

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17 Meta-omics in Detection of Silkworm Gut Microbiome Diversity

Mohanraj Ponnusamy, Chinnan Velmurugan Karthikeyan, and Babu Ramanathan

Abstract

Insect gut symbiotic microbiota plays an essential role in the growth, development, pathogenesis, and environmental adaptation of host insects. As such, the molecular and systems level analysis of insect gut symbiotic microbial community may aid in discovery of novel biocatalysts for biomass deconstruction and to develop innovative strategies for pest management. In this review, we focused on understanding the current knowledge on investigation of insect gut microbes, especially in silkworms, and their functional role in the insect gut environment. Genome analysis has emerged as a major tool to study the composition, function, and evolution of various microbiota. We have particularly explored the use of metagenomics and metaproteomics in the field of studying insect gut microbiota and the recent advances in this field toward exploring the insect gut symbionts.

Keywords

Insect gut symbiotic · Microbiota · Gut microbes · Metagenomics · Metatranscriptomics

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17.1 Introduction

Silkworm is a beneficial insect in silk industry, and silk production is primarily dependent on silkworm larval nutrition that is regulated by the midgut enzymes in food digestion (Moran 2007). Gut microbes of the insects, especially silkworms, play an essential role for the adaptation, biomass degradation, nutrient production, and compound detoxification (Shi et al. 2011). Influence or interference to these gut microbes due to internal and external factors could potentially affect the silkworm health including cause of serious diseases such as colony collapse disease (CCD) (Cox-Foster et al. 2007). The diversity of symbiotic gut bacteria varies widely according to different breeds of insects and environmental conditions.

The bacterial cultures can be identified using a variety of biochemical tests like specific enzymes secreted by different genera of bacteria (Madigan et al. 2015). Molecular techniques provide an opportunity to describe the microbial diversity independent of culturing live bacteria, which is an important adjunct to the culture-dependent approach (Urakawa et al. 1999). The most common molecular approach to explore microbial diversity and to identify uncultured bacteria is by using 16S rRNA gene (16S rDNA). The gene is approximately 1500 bp long and codes for the smaller subunit of ribosomal RNA of prokaryotes. The 16S rRNA gene combines highly conserved and variable regions as a useful tool in identification of bacterial taxa by their sequences.

The recent advances in "omics" technologies have enabled us to explore microorganism communities in an unprecedented way (Chen et al. 2015; Cheng et al. 2017; Grob et al. 2015; Su et al. 2016). The high-throughput metagenome and metaproteome analysis have helped us in speeding up molecular level investigations in conjunction with use of complementary data annotation and high-throughput functional screening (Grob et al. 2015; Hongoh et al. 2008). Efforts have been dedicated in discovery of novel enzymes, pathways, and organisms for various applications (Green et al. 2008; Roussel et al. 2008). Meta-omics sequencing has also become an important strategy for exploring biomass-degrading mechanisms in other insects such as wood-feeding insects (Warnecke et al. 2007) and lower termites (Hongoh et al. 2008). Earlier studies have also shown the symbiotic bacteria and protozoa in the hindgut of termite that play an important role in hydrolysis of cellulose and hemicelluloses (Tokuda and Watanabe 2007; Warnecke et al. 2007; Wheeler et al. 2007; Zhou et al. 2007). These analyses not only revealed a diverse group of bacteria covering 12 phyla and 216 phylotypes but also led to identification of more than 100 candidate glycoside hydrolases. The advancements in "meta" approaches aided us to better understand the microbial diversity and have been driven by increasing demands for biocatalysts for industrial applications (Su et al. 2016).

In this chapter, we review the metagenomics and metaproteomics tools that have been used to study the diversity of gut microflora in silkworm larvae and identification of potential factors that could play a role in contributing toward silkworm midgut ecosystem, silkworm adaptation, nutritional values, silk production, and quality.

17.2 Diversity of Gut Microflora in Silkworm and Other Insects

Insects are the most diverse with over a million different species found almost in every habitat (Green et al. 2008). Due to their widespread distribution, insects are inevitably associated with an extremely large variety of microscopic life forms, including viruses, bacteria, fungi, protozoa, nematodes, and multicellular parasites. Although some of these microorganisms exhibit a rather wide host range, many associations are highly specialized and involve not only certain insect species but also particular life stages of the insect host. In silkworm, gut microbes especially several bacterial genera and species with respect to seasons and breeds have been isolated and documented (Madigan et al. 2015).

The insect gut provides a suitable habitat for bacteria, and in many insect species, the gut possesses different types of bacteria, which are transient and do not remain in the gut during all life stages. However, in some insects, a variety of permanent microorganism's habitats and they supply essential nutrients to their host (Urakawa et al. 1999). Bacterial diversity of the soil-dwelling collembolan insect *Folsomia candida* has been reported to be predominantly associated with *Erwinia amylovora*, *Staphylococcus capitis*, and *Pantoea agglomerans*. It was also shown that various *Escherichia coli*-borne plasmids could be effectively transferred to different gut bacteria of *F. candida* indicating that insect gut is an important environment for horizontal gene transfer (Engel and Moran 2013). Termite and cockroach gut houses a complex microbiota ranging from protozoan spirochetes, gram-positive and gram-negative bacteria, archaea, and yeast (Urakawa et al. 1999). Lactic acid-producing bacteria are considered important for the ecological balance in the termite gut. Lactic acid metabolites serve to maintain homoeostasis of the bacterial community in the termite gut and also act as antagonists against colonization of the gut by opportunistic bacteria and maintain the micro-oxic zones within the gut environment (Potrikus and Breznak 1980; Tokuda and Watanabe 2007).

Many insect species derive their gut microbiota from the surrounding environment such as the phylloplane of food plants. For instance, aphid's gut microbes share a common ancestry with intracellular symbionts and bacteria ingested from food plants. Presence of bacteria in the gut of mulberry silkworm (*Bombyx mori*) has been reported, and the plant epiphyte *Erwinia herbicola* in the gut of *B. mori has been* shown that they were able to grow and survive in the gut environment (Tokuda and Watanabe 2007). The silkworm bivoltine breed NB4D2 harbored higher load of bacteria (16.5×10^4 cfu g^{-1}) in comparison with Pure Mysore and PM x NB4D2. Gut microflora from pure silkworm races revealed that bivoltine breed CSR2 recorded significantly higher load of bacteria than that of multivoltine breed Pure Mysore (Ramesha et al. 2012). The gut symbionts are vertically transmitted through host generations and play important role in growth, survival, and reproduction of the host insects.

17.3 Functional Role of Gut Bacteria

17.3.1 Nutrition

Nutritional symbioses in microorganisms and insects evolve when a major component of the insects' diet lacks sufficient quantities of specific nutrients or when nutrients present in the diet are inaccessible because the insect lacks the requisite metabolic tools to fully digest their food. Microbial-based nutritional symbioses are particularly well studied in insects with highly restricted diets of limited nutrition (e.g., blood, plant sap, wood) (Moran 2007). In these systems, bacteria or fungi help in nitrogen processing, sulfate assimilation, and fatty acid metabolism and help to contribute deficient sterols, vitamins (especially B-vitamin groups), digestive enzymes, and essential amino acids to their insect hosts (Donini et al. 2017; Sudakaran et al. 2012; Urakawa et al. 1999). Microorganisms possess metabolic properties that are absent in host insects, and they act as "microbial brokers" in overcoming biochemical insufficiencies of phytophagous insects. Aphids feeding on plant sap having lower concentrations of essential amino acids rely on their gut bacteria to provide the required amino acids and could be a major cause for CCD (colony collapse disease) (Cox-Foster et al. 2007).

In silkworm, vitamin B_{12} content in *B. mori* due to the presence of actinomyces changes according to the stage of development with maximum in larvae, minimum in eggs, and greatest in the Malpighian tubules. The synthesis of cobalamine was related to the actinomyces in digestive tubes (Salem et al. 2013). The bacteria inhabiting the gut of silkworm were found to be elaborating amylase, caseinase, gelatinase, lipase, and urease. The highest percentage of isolates was protease producers followed by lipid and polysaccharide splitters. These findings collectively indicate that the bacterial flora play an important role in digestion of ingested food material (Nangia et al. 1999; Ponnuvel et al. 2003; Ramesha et al. 2012; Shi et al. 2011).

17.3.2 Digestion

Insects, just like other organisms, will require to accommodate microbes in their alimentary canal systems which aid in digestion and also contribute to the nutrition of the host. The role of bacteria in nitrogen fixation in the gut of termites has been shown, and microbes can be able to detoxify plant-derived compounds such as flavonoids and alkaloids. Digestive enzymes of some insects are derived from their microbiota illustrated well in the hindgut fermentation system of termites and cockroaches (Nakashima et al. 2002; Tokuda and Watanabe 2007; Wheeler et al. 2007). The gut microbes rapidly adapt to changes in the insect diet by induction of enzymes and through population changes in microbial community. The midgut of insects is composed of epithelial and regenerative cells which are responsible for digestion, secretion, and absorption. The role played by microorganisms in insect digestion is highly significant. Microorganisms ferment the wood, and without them, the insect larvae would be unable to utilize the cellulose of the wood (Nakashima et al. 2002;

Su et al. 2016). Microorganisms supply essential vitamins and other substances hence changes a poor diet into an adequate nourishing diet. Furthermore, ingested microorganisms liberate enzymes that remain active in the gut surroundings and thus expand or extend the digestion and metabolic capabilities of organisms that harbor them. Additionally, microbial products play subtle roles in the life of the insect, being involved in the digestion of refractory food and detoxification of secondary plant compounds (Geigenberger et al. 2017; Jiang et al. 2015; Zhou et al. 2007).

In silkworm, the enzymes amino peptidase, β-glucosidase, alkaline phosphatase, and ATPase were reported to be found in microvilli (brush borders) in midgut cells of *B. mori* and thus contributing in efficient food digestion (Guo et al. 2016). Enzymes such as cellulase (β-endoglucanase, cellobiohydrolase/FPcellulase), xylanase, and pectinase are of exogenous microbial origin, while enzymes including amylase and β-glucosidase are produced endogenously (Adlakha et al. 2011; Shi et al. 2011; Zhou et al. 2007). In *B. mori*, the load of cellulolytic bacteria increases with cellulose or hemicellulose in their diet. Endogenous α-amylase from the midgut of *B. mori* is shown to function best at pH 9.3 and was found to have an action pattern similar to porcine pancreas amylase. The high pH of the gut might be an adaptation of leaf-eating lepidopteran insects for digesting hemicellulose, for which the enzymes are usually provided by the midgut existing microbiota (Giri et al. 2017).

The enzyme cellulase responsible for cellulose digestion in eri silkworm *Samia cynthia ricini* larvae was found to be dispersed in the foregut (15–18%), midgut (56–63%), and hindgut (20–29%). Removal of gut flora and fauna by feeding an antibiotic did not affect the activity of the enzyme in the foregut but led to 14–19% and 22–30% decreases in the midgut and hindgut, respectively. It indicates that *P. ricini* larvae synthesize their own cellulose in addition to using the gut microorganisms for digestion. This shows the abundance of microorganisms in both the midgut and hindgut which are responsible for cellulase secretion and partially involve in the digestion of cellulose (Nakashima et al. 2002; Shi et al. 2011; Wenzel et al. 2002; Wheeler et al. 2007).

17.3.3 Immunity and Protection

Insect adaptability to adverse conditions could be attributed to the versatile role played by their gut microflora. It has been shown that the presence of diazotrophic bacteria in insect gut helps in nitrogen uptake. On the other hand, the production of indole derivatives and siderophores by *Pseudomonads* in the gut of *Plutella xylostella* was shown to have antagonistic effect toward entomopathogens. Production of extracellular chitinase has been shown to help in maintaining the physical property of peritrophic membrane (Dubey et al. 2016; Engel and Moran 2013; Vogel et al. 2008). In vitro inhibition of conidial germination of *B. bassiana* and *M. anisopliae* by gut bacteria including *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Streptomyces noursei* was reported in native breeds of *B. mori*. Production of

antibacterial/antifungal metabolites by the gut actinomycete *S. noursei* that inhibits the growth of bacteria and fungi was also evident in Indian silkworm breeds (Fruttero et al. 2016).

Lipase isolated from silkworm larval alimentary canal showed strong antiviral activity against *Bm*NPV, providing evidence that digestive juice might play an important role during peroral infection with *Bm*NPV. The understanding of the diet-derived intestinal bacterial community might yield insight into the relationship between gut bacteria and disease resistance of the silkworm (Giri et al. 2017; Ponnuvel et al. 2003). The gut bacteria may also resist the colonization of invading pathogens by production of a series of secondary metabolites. To note, hemolymph has a key role in eliciting innate immune responses, which is triggered when pathogens enter into silkworm. Microbes can also play other roles such as preventing the colonization of pathogenic microbes by either mass action or active involvement in immune reactions. The microbes may synthesize various compounds and small molecules that can be used by the insects which increases fitness in extreme abiotic environments and provides protection from natural enemies such as predators and parasitoids (Fruttero et al. 2016; Miyashita et al. 2015).

17.4 Meta-omics in Insect Gut Diversity Studies

The recent advances in "omics" technologies have enabled us to explore microbe communities in an unprecedented way (Muturi et al. 2017; Peterson and Scharf 2016; Su et al. 2016). The high-throughput metagenome and metaproteome analysis of gut microbes may allow distinct molecular level investigation of chemical and biological processes. The data annotation and high-throughput functional screening techniques enable us to identify novel catalysts that can be identified from bacterial strains to be used for bioremediation, biomass processing, and bioproduct synthesis (Hongoh et al. 2008; Warnecke and Hugenholtz 2007). In the past two decades, much effort has been dedicated to exploring the components of microbial communities from different niches at the molecular, organism, and ecological level to discover novel enzymes, pathways, and organisms for various applications (Green et al. 2008; Roussel et al. 2008). For instance, metagenome and metaproteomics sequencing have also become important approaches for exploring biomass-degrading mechanisms in wood-feeding insects.

Several studies have been carried out to reveal symbionts in the midgut and hindgut of wood-feeding higher and lower termites (Hongoh et al. 2008; Warnecke and Hugenholtz 2007; Warnecke et al. 2007). A number of studies have also indicated that symbiotic bacteria and protozoa in the hindgut of the termite play an important role in the hydrolysis of cellulose and hemicelluloses (Nakashima et al. 2002; Tokuda and Watanabe 2007; Wheeler et al. 2007; Zhou et al. 2007). These studies not only revealed a diverse group of bacteria covering 12 phyla and 216 phylotypes but also led to the discovery of more than 100 candidate glycoside hydrolases. In addition, a vast knowledge has been shed in identifying other important functions of symbiotic microbiota, including hydrogen metabolism, carbon dioxide-reductive

acetogenesis, and nitrogen fixation. Overall, the development of omics studies, especially metagenomics and metaproteomics, over the past decades has been focused on the better understanding of microbial diversity and function in the eco-environment and has been driven by increasing demands for biocatalysts and biomolecules for applications such as biorefinery (Jiang et al. 2017; Schmeisser et al. 2007; Stepan'kov et al. 2017).

17.4.1 Metagenomics in Exploring Insect Gut Microflora

Insects can adapt to extremely diverse eco-environments, particularly the herbivorous insects that can exploit a wide range of plant species as food. Insect gut symbionts play an essential role in the insect adaptation to various food types, and they have been shown to play an important role in lignocellulosic biomass degradation, nutrient production, compound detoxification, and environmental adaptation (Moran 2007; Shi et al. 2011). Disrupting insect gut symbionts could significantly reduce the fitness of insects and can even cause serious diseases such as CCD. Metagenomics can serve as a tool to study these insect gut symbionts and their role in such environment (Cox-Foster et al. 2007). Insect gut symbionts were shown to be maternally inheritable from generation to generation, which suggests the symbiotic microbiota is a dynamic component of the competitive evolution between plants and herbivorous insects as well as a driving force for insect speciation (Moran 2007). Studies have highlighted several important features of some insect gut symbionts including their reduced genome size, convergent evolution, co-speciation, and complementary function with the host genome (Cheng et al. 2017; Cox-Foster et al. 2007; Donini et al. 2017; Eleftherianos et al. 2013; Engel and Moran 2013). Recent studies also expanded our understanding of the roles of insect gut symbionts in nonconventional functions like nitrogen recycling, reproductive manipulation, and pigment production and many other aspects related to insect fitness (Jiang et al. 2017; Muturi et al. 2017; Ojeda et al. 2017; Stepan'kov et al. 2017).

Despite the progress toward understanding insect-symbiont relationships, there is still much to be learned especially with regard to facultative symbionts. Moreover, limited research has focused on comparing the gut symbionts from insect species that specialize on different food sources. For this reason, systematic comparison of gut enzyme activities and microbial diversity in several insect species relevant to biotechnology applications is a priority focus area (Cox-Foster et al. 2007; Shi et al. 2011). The comparison of the microbial community of gut symbionts from wood borer, silkworm, grasshopper, and cutworm using DGGE (denaturing gradient gel electrophoresis) has also revealed significant differences in symbiotic community correlating with food adaptation (Shi et al. 2011). However, an in-depth understanding of the eco-evolutionary adaptation to food types requires metabolic and phylogenic analysis, which cannot be otherwise offered by traditional approaches like DGGE. Several earlier studies in comparative biology of symbionts from different insect species were either carried out with DGGE or focused on one or few

symbiotic species. Compared to those conventional techniques, new platforms like metagenomics could help define the function of symbionts in the food adaptation of insects and promote discovery of biocatalysts for biotechnology applications.

Metagenome analysis has emerged as a major approach to study the composition, function, and evolution of various microbiota. Metagenome analysis and metabolic reconstruction of gut symbiotic microbiota in several insects have revealed potential functionality in these microbiomes that might be required for biomass degradation, nutrient synthesis, and other functions essential to the insect (Muturi et al. 2017; Nangia et al. 1999; Nelson 2008). In addition, these studies also highlighted the potential for biotechnology applications of these insect gut symbionts, as many potential glycosyl hydrolase (GH) family enzymes have been identified from the insect gut (Warnecke and Hugenholtz 2007). Furthermore, studies have also revealed the potential complementary function between the host and symbiont enzymes for highly efficient biomass degradation (Adlakha et al. 2011; Grob et al. 2015; Shi et al. 2011). However, many studies have focused on the metagenome sequencing of symbionts in single insect species or the same symbiont in different insect species (Hongoh et al. 2008; Wheeler et al. 2007). Very few studies have systematically compared the metagenomes of symbiotic microbiota from insect species with distinctly different diets, environmental adaptations, or life histories, and this type of comparative metagenomics strategy has the potential to substantially improve our understanding of the adaptive significance of insect gut symbionts for insect diet specialization as well as facilitate the discovery of novel biocatalysts for biorefinery applications (Cheng et al. 2017; Muturi et al. 2017; Peterson and Scharf 2016; Su et al. 2016). Metagenomics is a culture-independent strategy involving high-throughput functional screens and sequence-based analysis of metagenomic libraries which have led to the identification of novel microbial genes and their products from soil, seawater, and other environments. Metagenomic analysis of insect-associated microorganisms has yielded an understanding of biosynthetic pathways for secondary metabolites from associated biota, for example, xylanases, with unusual primary sequences and novel domains of unknown functions in microbiota (Donini et al. 2017; Luengo et al. 2001; Shi et al. 2011).

17.4.2 Metaprotoeomics for Revealing Insect Gut Symbionts

The study of metaproteomics of insect gut symbionts is like any metagenome sequencing project where genome sequencing is the first step toward a comprehensive understanding of composition, dynamics, and function of insect gut symbiotic microbiota. The sequence itself might not be enough to understand the expression and the dynamic changes of the system. Post-genomic molecular approaches such as proteomics will allow us to study the ultimate functional products of genes/genomes and derive the function and dynamics of insect gut system. The collective study of all proteins in microbial communities, such as those in insect gut, has been documented in insects with insights into the functional relationships (Chen et al. 2015; Grob et al. 2015). Metaproteomics allows the measurement of gene

expression from the perspective of presence and abundance of translated proteins and is a gel-free strategy. Ideally, the protein samples are subjected to the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by a mass spectrometry (MS)-based protein identification. The MS techniques that are used for protein identification include matrix-assisted laser desorption ionization (MALDI), liquid chromatography (LC), and electrospray ionization (ESI). MALDI is usually coupled with time-of-flight (TOF) mass analyzer, while LC and ESI are coupled with a variety of mass analyzers (Grob et al. 2015).

One of the earlier yet popular approaches in metaproteomics strategy was a MudPIT (multidimensional protein identification technology)-based shotgun proteomic strategy (Delahunty and Yates 2007). In this, the total protein from a sample was first digested by protease into a peptide mixture, and then the peptide mixture was further separated by multidimensional LC. The separated peptides were further analyzed by MS/MS for protein identification as aforementioned. Despite the vast advancements in the proteomics field, the application of metaproteomics in the analysis of insect gut symbiotic microbiota is still very limited to certain insect gut biota. In silkworm, the midgut is a barrier to foreign substances during food digestion, and it has been found that some proteins such as lipase and SP-2 in the midgut have antiviral activity against *B. mori* nuclear polyhedrosis virus (BmNPV) (Ponnuvel et al. 2003). Metaproteomics have been used to reveal the molecular mechanisms for nutrition digestion and midgut-derived defense of the silkworm larval midgut (Chen et al. 2015; Guo et al. 2016). However, the studies are in its early phases as there are many unknown microbiota that may colonize and contribute for the silkworm gut environment.

17.5 Conclusion

Microorganisms living in insect gut play a crucial role in the adaptation, growth, and development of the insect hosts. The identification and molecular analysis of insect gut microbes can enable us to develop novel strategies for industrial product development and effective utilization of by-products and facilitate us with the best pest management practices. Silkworm undergoes radical morphological variations upon metamorphosis, and a key challenge in studying gut microflora is the ability to access different genomes and the corresponding protein profiles. Employing the appropriate tool such as metagenomics in combination with metaproteomics strategy may help to bridge the gap between the unknowns of silkworm gut microbiota and their importance in the silkworm gut ecosystem.

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