Joginder Singh · Deepansh Sharma
Gaurav Kumar · Neeta Raj Sharma
Editors

Microbial Bioprospecting for Sustainable Development

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Part I

Microorganisms for Sustainable Agriculture and Environmental Applications

Check for updates

1 Small at Size, Big at Impact: Microorganisms for Sustainable Development

Nasib Singh, Joginder Singh, and Karan Singh

Abstract

From being the first life originated on Earth ~3.8 billion years ago to the present time, microorganisms have enormously impacted the human, animal, and plant's lives and global biogeochemical cycles in one way or another. These are widely distributed in almost all habitats and ecosystems on Earth, including the most hostile and extreme habitats which are otherwise uninhabitable to other organisms. Domain *Bacteria* and *Archaea* are composed entirely of prokaryotic microorganisms, whereas eukaryotic microbes, viz., fungi, algae, protozoa, slime molds, and water molds, belong to domain *Eukarya*. Archaea and bacteria represent the majority of life-forms on our planet. Recent estimate predicts 10^{11}–10^{12} microbial species on Earth of which 99.9% microbial species are yet to be cultured in the laboratory. Ocean, soil, rhizosphere, human gut, animal body, etc. are some of the most densely populated microbial habitats. Microorganisms are excellent model organisms for the study of metabolism and genetics at cellular level. Considered as Earth's greatest chemists, microorganisms have unparalleled metabolic capabilities, extraordinary adaptability, and remarkable survival strategies which undoubtedly make them the most successful living creatures. Most microbes are beneficial to humans, plants, and animals. These contribute significantly to ensure the quality of human life and in sustaining life on our planet. Microbes have established ecologically important symbiotic and

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nonsymbiotic associations with themselves, humans, plants, ruminants, vertebrates, and invertebrates. Incomparable importance of microorganisms led to the origin of concepts of microbiome, hologenome, and superorganism. Microorganisms offer numerous biotechnological compounds for human, animal and agriculture, and environment sustainability. These are the source of numerous bioproducts like antibiotics, biopharmaceuticals, single-cell proteins, organic acids, biofertilizers, biopesticides, enzymes, pigments, vitamins, biofuels, biocement, and many more. Harnessing microbial capabilities is undoubtedly the best possible sustainable solution to ever-increasing challenges of balanced diet, clean air, water, energy, medicine, and healthy environment.

Keywords
Microbial diversity · Sustainable development · Microbiome · Rhizosphere · Nitrogen fixation · Probiotics · Microbial cell factories · Biopharmaceuticals

1.1 Introduction

Microorganisms (or microbes) are microscopic living organisms invisible to unaided eyes. Being the earliest, extremely adaptable, unprecedentedly diverse, and most successful living organisms on the Earth, microbes have extensively impacted the lives and geochemical processes in one way or another across our biosphere. Bacteria, archaea, fungi, algae, and protozoa are the major categories of microorganisms. Viruses (acellular in nature) and certain life stages of helminth parasites are also considered in the scope of microbiology. Despite being the earliest lifeforms originated on the Earth about 3.8–4.2 billion years ago (Bunge et al. 2014; Weiss et al. 2016), microbial existence was only proved in the seventeenth century by a Dutch tradesman Antonie van Leeuwenhoek with the help of handmade simple microscope. He observed bacteria from his own body and termed them "animalcules." Famous microbiologist Dr. Carl Woese had termed the microbial world as "Biology's Sleeping Giant" (Woese 1998). Microbes are ubiquitously present in almost every ecological niche that can exist on the Earth. The complex microbial communities of mammals and plants are termed microbiome. Microbe's adaptability to extreme environmental conditions and habitats is incomparable in the entire biological world. The field of microbiology has since grown in leaps and bounds with great contributions of stalwarts, viz., Louis Pasteur, Robert Koch, Hans Christian Gram, Julius Petri, Paul Ehrlich, Alexander Fleming, Joshua Lederberg, Elie Metchnikoff, Sergei Winogradsky, Martinus Beijerinck, Thomas Brock, Carl Woese, Craig Venter, and many more (www.asm.org). Our current understanding of complex cellular processes, gene functions, and metabolic machinery is significantly contributed and impacted by microorganisms which served as model organisms. Extensive research on *E. coli*, *Saccharomyces cerevisiae*, and bacteriophages offered deeper insights into cellular processes in general and human biology in particular.

1.2 Origin of Life and Microbes: Billion-Years-Old Connection

Prof. Carl Woese proposed three domains of life, i.e., *Bacteria*, *Archaea*, and *Eukarya* on the basis of ribosomal RNA sequence analyses (Woese and Fox 1977; Woese et al. 1990). This marked the birth of a new group of microbes called archaea which are phylogenetically distinct from bacteria and eukaryotes. The available evidences point toward the origin of life about 3.5–3.8 billion years ago amidst extreme and inhabitable environmental conditions (Bekker et al. 2004; Hug et al. 2016). Our most likely ancestor was a single-celled microscopic organism, LUCA or last universal common ancestor (Weiss et al. 2016). Thriving in hydrothermal vents, LUCA was strictly anaerobic, N_2-fixing, CO_2-fixing, and H_2-dependent autotroph with a Wood-Ljungdahl pathway (Weiss et al. 2016). Recently, Hug et al. (2016) constructed a new tree of life using genome sequences of thousands of species including genomes of uncultured microbial species from three domains of life. Remarkably, this tree has 92 bacterial phyla and 26 archaeal phyla along with eukaryotic fungi, algae, protozoa, and other protists.

1.3 Diversity of Microbial World

The diversity and ubiquity of microorganisms is enormous and remarkable. Their exceptional ability to colonize almost all possible ecosystems and extraordinary metabolic capabilities makes them the most versatile and successful inhabitant on our planet. These are found in soil (up to 3 km deep), ocean, freshwater bodies (lake, river, ponds), air, rocks, desert, polar ice, oil fields, underground coal mines, underwater hydrothermal vents, hot springs, acidic lakes, soda lakes, Dead Sea, human body, walls, rocks, animal body, compost piles, rumen, termite gut, corals, paddy fields, under deep pressure, anaerobic environments, sea anemones, tube worms (hydrothermal vents), insect guts, root nodules (symbiotic bacteria), plant cells (endophytes), plant roots (mycorrhiza), insect cells (endosymbionts), computer keyboards, subways/tunnels, smartphone, etc. (Brock 1967; Ley et al. 2006; Sirohi et al. 2012; Bunge et al. 2014; Clarke 2014; Canganella and Wiegel 2014; Mazard et al. 2016; Martin and Mcminn 2017). Several species of bacteria and fungi are also reported to survive inside the modules of space stations.

Unfortunately, our current knowledge on microbial diversity is limited, and major bottleneck is non-culturability of approximately 99.9% of bacterial and archaeal species (Bunge et al. 2014). Advances in 16S rRNA gene sequencing technologies have offered new opportunities to gain deep insights into microbial community structures, nutritional interactions, and novel functions. Several attempts have been made to accurately estimate and enumerate the microbial species and their diversity in various ecosystems. Bacterioplankton and phytoplankton (protists) contribute most to the Earth's biomass. Bacterioplankton, *Prochlorococcus marinus*, is the most abundant photosynthetic microbe on Earth (Dufresne et al. 2003). Similarly, *Pelagibacter ubique* is regarded as most abundant marine bacterium (Giovannoni et al. 2005). It has smallest cell volume, smallest genome, and smallest

ORFs among free-living cells. *Ostreococcus tauri*, a green alga, is a dominant photosynthetic eukaryote in water bodies (Derelle et al. 2006). Several unique aspects of microorganisms are described in Table 1.1. According to Pomeroy et al. (2007), microbial biomass is 5–10 times bigger than that of all multicellular marine organisms. Whitman et al. (1998) predicted 1.2×10^{29} prokaryotes in seawater and 2.6×10^{29} in the soil. Kallmeyer et al. (2012) gave higher estimate of 2.9×10^{29} microbial cells in subseafloor sediments. Recent estimate predicts 10^{11}–10^{12} microbial species on our planet (Locey and Lennon 2016). However, just 11,000 species belonged to 30 bacterial and 5 archaeal phyla have been isolated and validly classified so far (Gutleben et al. 2017). There are approx. 8.74 million species of eukaryotes existing on the Earth of which approx. 611,000 are fungi and approx. 36,400 are protozoa (Mora et al. 2011).

The gastrointestinal tracts of human and other mammals contain dense, complex, and unique microbial communities (Ley et al. 2006; Human Microbiome Project Consortium 2012). Human gut is one of the densest and complex ecosystems occupied by bacteria, archaea, fungi, protozoa, and viruses. This microbial consortium, also called gut microbiota, is highly dynamic and diverse (Ley et al. 2006; Mirzaei and Maurice 2017). The human gut harbors 10^2 cfu/g (proximal end) to 10^{11} cfu/g (distal end) bacteria (Donaldson et al. 2016). Similarly, human oral cavity is home to about 700 bacterial and 90 fungal species. The combined gene content of human microbiota is staggering 50–100 times the size of human genome. In general, human gut is dominated by bacterial phyla *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia*. The small intestine, in particular, is mostly inhabited by families *Lactobacillaceae* and *Enterobacteriaceae*, whereas the colon is dominated by the families *Bacteroidaceae*, *Prevotellaceae*, *Lachnospiraceae*, *Rikenellaceae*, and *Ruminococcaceae* (Donaldson et al. 2016). Apart from bacteria, human body also has archaea, fungi, protozoa, and viruses (Human Microbiome Project Consortium 2012; Mirzaei and Maurice 2017). In sharp contrast to generally accepted view of 10 microbial cells for each human cell, Sender et al. (2016) gave revised estimates of 3.8×10^{13} bacteria in the whole body of a 70-kg reference man having 3.0×10^{13} own body cells. The bacteria to human cell ratio is now estimated to be 1:1.3 which is much lower compared to earlier estimates. These estimates found only about 0.2 kg of bacteria in the whole human body, whereas NIH-Microbiome Project estimated 1–3 kg of bacteria in a normal human being. Further refinement and actualization in these estimates are expected in the near future.

Many microbes prefer company of a partner for survival and growth. The best known examples of these relationships are lichen and obligate insect endosymbionts. Lichens represent one of the oldest and most recognizable symbioses in nature. Recently, the existing theory of two-partner (ascomycete fungus and algae/cyanobacteria) lichen association has been challenged with the discovery of third symbiotic partner, a basidiomycete yeast *Cyphobasidium* in macro-lichen *Bryoria fremontii* (Spribille et al. 2016). Insect endosymbionts or nutritional symbionts reside in the cells of plant sap-feeding insects such as aphids, whiteflies, leafhoppers, and cicadas. These endosymbionts have extremely minimized genome size and provide essential amino acids and vitamins to insect hosts (Moran and Bennett

2014). One widely known example of such bacteria is *Wolbachia* (Lo et al. 2016). Interestingly, the obligate endosymbiotic bacterium carries minimal genetic contents. "*Candidatus* Nasuia deltocephalinicola" is characterized by smallest genome size (112,031 bp) and as few as 140 genes among all cellular organisms (Moran and Bennett 2014; Bennett et al. 2016; Table 1.1).

Viruses, the acellular microorganisms, infect cells from all three domains of life. These have significant ecological and genetic functions in the biosphere such as microbial population control, gene transfer, and genome novelty. Bacterial viruses (bacteriophages) are key in controlling bacterial numbers in aquatic ecosystems, acquiring genetic novelties through transduction, and also offer an affordable solution to tackle antibacterial drug resistance. Other acellular agents, i.e., animal viruses, human viruses, plant viruses, viroids (naked ssRNA), virusoids (ssRNA), and prions (proteinaceous infectious particles), are pathogenic to plants and animals. Their study is equally important from human health perspective, biomass turnover, and for developing strategies to tackle ever-increasing multidrug resistance in bacterial pathogens. Contrary to century-old accepted view of submicroscopic viral size, a giant virus was discovered in 2003 which can be seen under the light microscope (Aherfi et al. 2016; Colson et al. 2017). It was named *Mimivirus* and had huge dimensions as well as genome size. Later, Philippe et al. (2013) isolated giant micrometer-sized viruses, called *Pandoravirus*, having a genome and cell sizes bigger than some of bacterial species. In terms of size, *Pithovirus sibericum* is the largest virus known so far (Legendre et al. 2014). The various giant viral families/genera described so far include *Mimiviridae*, *Marseilleviridae*, pandoraviruses, faustoviruses, mimivirus virophages, *Pithovirus sibericum*, and *Mollivirus sibericum* (Aherfi et al. 2016; Colson et al. 2017). Giant viruses are not only larger (2–15 times) than traditional viruses, but these also have 50–250 times more genes (Aherfi et al. 2016). It is now a matter of debate and controversy whether these giant amoebal viruses represent the possible fourth domain of life or not.

1.4 Remarkable Survival and Adaptability of Microorganisms

Some fascinating microorganisms thrive or love to grow in extreme environmental conditions, which are generally considered inhospitable for life. Where no other life-forms can survive and grow, microorganisms flourish with ease to drive geochemical cycling on Earth (Pikuta et al. 2007; Poli et al. 2017). These organisms, called extremophiles, are found in polar ice, hot springs, hydrothermal vents, salt lakes, acid and alkaline habitats, and toxic wastes (Table 1.2). Acidophiles, alkaliphiles, thermophiles, hyperthermophiles, psychrophiles, halophiles, and barophiles are some examples of extremophiles. Moreover, some microbes grow at two or more extreme conditions and hence called polyextremophiles. Interestingly, extremophiles not only tolerate the extreme conditions, but they actually require these for their metabolism and growth cycles. Hyperthermophilic archaea are considered to be the earliest life-forms thrived on Earth and regarded as the most extreme microbes

Table 1.1 Some extraordinarily unique microorganisms across three domains of life

Microbial species	Domain	General category/phyla	Habitat	Unique characteristics	References
Armillaria solidipes	*Eukarya*	Fungi	Malheur National Forest, Oregon (USA)	Approx. 965 ha with an estimated age of 1900–8650 years (largest living organism on Earth)	Smith et al. (1992)
Caulerpa taxifolia	*Eukarya*	Green alga	Freshwater	World's largest single-celled organism (size in meters)	Ranjan et al. (2015)
Pelagibacter ubique	*Bacteria*	*Proteobacteria*	Ocean; in ultraoligotrophic habitats	Smallest free-living cell	Giovannoni et al. (2005)
				Length: 0.37–0.89 μm	
				Diameter: 0.12–0.20 μm	
				Volume: ~0.01 $μm^3$	
Thiomargarita namibiensis	*Bacteria*	*Proteobacteria*	Namibia (at the bottom of the ocean) "sulfur pearl of Namibia"	Largest bacterium on Earth (can be seen with naked eye)	Schulz et al. (1999)
				Diameter: 180–750 μm	
				Volume: $3–28 \times 10^6$ $μm^3$	
Pelagibacter ubique	*Bacteria*	*Proteobacteria*	Ocean; in ultraoligotrophic habitats	Most abundant heterotrophic bacterium in ocean	Giovannoni et al. (2005)
Prochlorococcus marinus	*Bacteria*	Cyanobacterium	Oceans, seas	Most abundant and smallest photosynthetic microbe on Earth (cell size 0.5–0.7 μm)	Dufresne et al. (2003)
Ostreococcus tauri	*Eukarya*	Green alga	Oceans, sea, coastal waters	Smallest free-living eukaryote (diameter 2–3 μm)	Derelle et al. (2006)
"*Candidatus* Nasuia deltocephalinicola"	*Bacteria*	*Proteobacteria*	Obligate insect endosymbiont	Smallest bacterial genome (112,031 bp)	Bennett et al. (2016)
Pelagibacter ubique	*Bacteria*	*Proteobacteria*	Ocean; in ultraoligotrophic habitats	Smallest genome (1,308,759 bp) and predicted ORFs (1354) for a free-living cell	Giovannoni et al. (2005)
Ktedonobacter racemifer	*Bacteria*	*Chloroflexi*	Soil	Largest bacterial genome (13,661,586 bp)	Chang et al. (2011)

Sorangium cellulosum strain So015 7-2	*Bacterium*	Myxobacterium	Soil	Second largest bacterial genome (13,033,779 bp)	Schneiker et al. (2007)
Encephalitozoon intestinalis	*Eukarya*	Microsporidia (Fungi)	Human parasite	Smallest eukaryotic genome (2.3 Mbp)	Corradi et al. (2010)
Pandoravirus salinus	–	Giant virus	Infect *Acanthamoeba castellanii*	Largest viral genome (length 1.0 μm; diameter 0.5 μm)	Philippe et al. (2013)
				Genome: 2,473,870 bp	
Pithovirus sibericum	–	Giant virus	Infect *A. castellanii*	Largest virus in terms of size (length 1.5 μm; diameter 0.5 μm)	Legendre et al. (2014)
Trichomonas vaginalis	*Eukarya*	Protista	Obligate human parasite	Largest microbial genome (176,441,227 bp)	Carlton et al. (2007)
				Highest number of protein-coding genes for a eukaryotic microbe (~60,000)	

Table 1.2 Some record-holder extremophilic microorganisms adapted to grow and survive under most extreme and unusual habitats on Earth (Compiled from Pikuta et al. 2007; Clarke 2014; Lebre et al. 2017; Krüger et al. 2017)

Microorganism	Domain	Growth parameters	Habitats	Remarks
Methanopyrus kandleri strain 116	*Archaea*	122 °C (survive at 130 °C up to 3 h)	Kairei hydrothermal vent field, Central Indian Ridge (depth 2450 m)	Record for growth at highest temperature
Geogemma barossii strain 121	*Archaea*	121 °C	Hydrothermal vent, Juan de Fuca Ridge, Pacific Ocean	Survive at 130 °C for 2 h
Pyrolobus fumarii	*Archaea*	113 °C	Hydrothermal vent, Atlantic Ocean	Extreme thermophile
Pyrodictium abyssi	*Archaea*	110 °C	Hydrothermal vents	Extreme thermophile
Geothermobacterium ferrireducens	*Bacteria*	100 °C	Yellowstone National Park, USA	Record for growth at highest temperature (for domain *Bacteria*)
Cyanidium caldarium	*Eukarya*	55–56 °C	Yellowstone National Park, USA	Most thermo-tolerant eukaryote
Nitzschia frigida	*Eukarya*	−8 °C	Brine in frozen sea water	Record for growth at lowest temperature
Natrialba hulunbeirensis; *Natronolimnobius aegyptiacus*	*Archaea*	Na^+ 3.4–4.5 M, pH 9.0–9.5 Temp. 50–55 °C	Soda lakes	Halophilic alkalithermophiles
Deinococcus radiodurans	*Bacteria*	Resistance to radiations, oxidizing agents, and mutagens	Meat, animals, hot springs	World's toughest bacterium. Can tolerate up to 1500 kilorads γ-radiations
Picrophilus torridus *Picrophilus oshimae*	*Archaea*	pH 0.07	Hot spring in Hokkaido, Japan	Most acidophilic living organisms
Anaerobranca gottschalkii	*Bacteria*	Survive at 65 °C, pH 10.5	Hot inlet of Lake Bogoria, Kenya	Thermoalkaliphilic bacterium
Aspergillus penicillioides *Xeromyces bisporus*	*Eukarya*	Water activity 0.585–0.637	Raisins, Australia and Antique wood, Thailand	Most extreme xerotolerant fungi
Thermococcus piezophilus	*Archaea*	$Pressure_{max}$: 130 MPa	Ocean bottom	Extreme piezophilic archaeon

(Weiss et al. 2016). The current high-temperature limit for growth is blistering 122 °C exhibited by hyperthermophilic methanogen *Methanopyrus kandleri* strain 116 (Takai et al. 2008; Clarke 2014). *M. kandleri* could remain viable for up to 3 h at 130 °C. Hyperthermophilic archaeon, *Geogemma barossii* (also called strain 121) isolated from a hydrothermal vent, is another extreme thermophilic microbe which grows at 85–121 °C and can survive at 130 °C for up to 2 h (Kashefi and Lovley 2003). *Pyrolobus fumarii* is another archaeon capable of growth at 106 °C. *Natranaerobius* is a polyextremophile which shows optimal growth above pH 9.5, 69 °C, and 4 M Na^+ salt concentration (Canganella and Wiegel 2014). In case of higher eukaryotes, growth is seldom reported above 60 °C except some nematodes, algae, and polychaetes (Clarke 2014). Unicellular alga, *Cyanidium caldarium*, is reported to complete its life cycle at 55–56 °C. However, other eukaryotes usually not grow but survive in this temperature range. Some other extremophilic microbes are discussed in Table 1.2. Recently, the Extreme Microbiome Project (XMP) is launched to explore the microbial diversity of several extreme habitats, viz., acidic hypersaline ponds (Australia), Lake Hillier (Australia), "Door to Hell" crater (Turkmenistan), ocean brine lakes (Gulf of Mexico), deep ocean sediments (Greenland), permafrost tunnels (Alaska), ancient microbial biofilms (Antarctica), blue lagoon (Iceland), and toxic hot springs (Ethiopia) (Tighe et al. 2017).

Deinococcus radiodurans (nicknamed Conan the Bacterium by NASA) is a Gram-positive, extremely radiation-resistant bacterium that can tolerate ionizing radiations up to 3000 times more than human cells and survive in extreme heat, desiccation, acidity, and oxidative damages (Cox and Battista 2005; Slade and Radman 2011). It is rightly regarded as the "world's toughest bacterium" due to its remarkable capacity to repair DNA double-strand breaks. Space environment is an extremely hostile environment characterized by microgravity, intense radiation, extreme temperatures, and high vacuum (Horneck et al. 2010; www.nasa.gov). Survival and growth in such conditions is one of the toughest challenges for microorganisms. Remarkably, two species of lichens (*Rhizocarpon geographicum* and *Xanthoria elegans*) remained viable for 2 weeks in outer space (Horneck et al. 2010). Dust samples from Russian segment of International Space Station found to harbor 80 bacterial and 1 fungal species (Mora et al. 2016). More than 90 species of microorganisms (including bacteria, archaea, fungi, algae, and viruses) have been to outer space for experimental research studies of NASA and other space agencies (www.en.wikipedia.org. accessed 12 June 2017). Recently, a bacterium was isolated from high-efficiency particulate arrestance filter system of the International Space Station and named *Solibacillus kalamii* (Checinska et al. 2017) in honor of Dr. A.P.J. Abdul Kalam, the former President of India.

1.5 How Microbes Affect Nature and Life

Microbial communities play an essential role in all major biogeochemical cycles on our planet and drive the carbon, nitrogen, phosphorus, and other nutrient cycling. Microbes support and sustain the very existence of life on the Earth. Gilbert and

Neufeld (2014) have rightly stated that "microbes sustain life on this planet" and "life would become incomprehensibly bad" in the absence of microbes. Although prokaryotic microorganisms have tiny size and minimal cell volume, yet they contribute huge biomass in marine and aquatic ecosystems. Cyanobacteria (formerly called blue-green algae) are photosynthetic prokaryotes which are responsible for the present oxygen-rich atmosphere of Earth (Bekker et al. 2004; Mazard et al. 2016). Algae and cyanobacteria are primary producers of oxygen and fix CO_2 to generate food for other organisms. Similarly, phytoplanktons (unicellular protists-algae, diatoms) and protozoa also play significant role in sustainability of food chains and food webs. Ocean is the largest ecosystem on Earth harboring phenomenal microbial diversity and huge microbial biomass. One liter of ocean water harbors more than 10 billion organisms, mainly prokaryotes, protists, viruses, and zooplanktons (www.embl.de). The first life-forms have their origin in ocean, and it is this ecosystem which sustains life on our planet. Billions of trillions bacteria (cyanobacteria, methanogens), viruses, and algae (phytoplanktons) constitute the base of food chain, absorb CO_2, and release O_2 into the atmosphere. Further, methanogens play a major role in primary production in deep-sea ecosystems (deep-sea hydrothermal areas and sediments). Microorganisms remained an essential part of human life either through their natural activities or by producing recombinant pharmaceuticals. These have the capabilities to help mankind in tackling the newer challenges of food, energy, and clean water in a sustainable manner while maintaining and improving the health of our ecosystems.

1.6 Human Microbiome

Human microbiome represents the symbiotic, commensal, and pathogenic microbial communities (bacteria, archaea, fungi, viruses, and protozoa) present on or inside human body. The NIH-funded Human Microbiome Project was initiated in 2007 at a cost of $173 million, and its findings have revealed interesting facts about human body ecosystem (Human Microbiome Project Consortium 2012). This massive project characterized the microbial communities in over 200 healthy volunteers (from 18 different body sites) and has generated about thousand reference genomes from human microbiota (Fodor et al. 2012). Its findings revealed that human body harbors more microbial cells than own cells, and the millions of genes expressed by the microbiome dwarf the 20,500 genes expressed by our genome (Proal et al. 2017). The discovery of the human microbiome marks one of the most important milestones in the history of science. Vast ecosystems of bacteria, viruses, bacteriophages, and fungi persist in every individual (Proal et al. 2017). Every hour, human body shed approx. 30 million bacteria into its surroundings (Qian et al. 2012). The most prominent role is played by gut microbiota which assist in the metabolism of indigestible polysaccharides, produce vitamins and amino acids, provide protection against colonization of gut by pathogens, enhance the host's response to pathogen invasion, and potentiate the immune system (Arnold et al. 2016; Mirzaei and Maurice 2017). Human microbiota can be utilized for the treatment and management

of infectious and chronic diseases, discovery of novel antibiotic-producing microbes, and improvement of general health in what could be called the most sustainable solution for human or public health issues. Recent research showed that microbiome of an individual can be utilized as unique identification marker for resolving criminal cases (Hampton-Marcell et al. 2017).

1.7 Microbe-Plant Interactions

Microorganisms living in the soil usually establish beneficial relationships with several species of leguminous and non-leguminous plants. This microflora, called rhizosphere microbiota, is key to plant fitness and soil sustainability. Rhizobia are rod-shaped Gram-negative plant growth-promoting rhizobacteria (PGPR) which perform biological nitrogen fixation (BNF) by forming root nodules in leguminous plants such as soybean, faba bean, pea, common bean, cowpea, lentils, chickpea, etc. BNF is carried out symbiotically by few genera of rhizobia, viz., *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Allorhizobium*, and *Azorhizobium* (Mus et al. 2016; Remigi et al. 2016). In addition, several free-living PGPR, i.e., *Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Klebsiella*, and *Azomonas*, also perform BNF in the soil. Most leguminous plants and some non-legume trees (*Parasponia*, *Casuarina*, *Datisca*, and *Alnus*) have nitrogen-fixing nodules formed by rhizobia and *Frankia*, respectively (Mus et al. 2016). Apart from nitrogen fixation, several species of microorganisms also promote plant growth in various ways. These include *Arthrobacter*, *Agrobacterium*, *Burkholderia*, *Chromobacterium*, *Caulobacter*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Serratia*, *Micromonospora*, *Streptomyces*, and *Thermobifida* (Udvardi and Poole 2013; Ahemad and Kibret 2014; Mus et al. 2016; Remigi et al. 2016). *Azolla* is heterosporous pteridophyte fern commonly cultivated in paddy fields in Asia for centuries as a companion crop. It harbors a N_2-fixing cyanobacterium, *Nostoc azollae*, and hence considered a sustainable natural source of nitrogen (Kollah et al. 2016). Some fungi establish associations with roots of higher plants to form mycorrhizae. Such association is beneficial not only for both the partners but also increases fertility and organic richness of the soil or aquatic ecosystems. Arbuscular mycorrhizal associations help the plants to obtain sufficient supplies of phosphorus from the rhizospheric soils (Igiehon and Babalola 2017). PGPR and fungi promote plant growth and development through nitrogen fixation, release of plant growth promoters, mineralization, phosphate uptake, and increased access to water. BNF, being a nonpolluting and a cost-effective bioprocess, has potential to decrease our dependence on chemical fertilizers, reduce environmental pollution, and minimize the cost of agriculture production. Applications of PGPR as bio-inoculants in the form of biofertilizers, rhizoremediators, phytostimulators, biopesticides, biofungicides, and bioherbicides are considered an eco-friendly and sustainable strategy for ecologically sustainable management of agricultural ecosystems. It will not only increase the productivity of soil systems but will also afford protection against abiotic stresses (salt, heavy metals), weeds, insects, and fungal pathogens. PGPR inoculants are advantageous for

agriculture systems especially in developing countries due to minimal investment cost involved and least damage to the environmental health. Biological research efforts must be directed toward introduction of symbiotic BNF into non-legume crops, cereals (wheat, maize, rice), and horticultural and medicinal plants.

1.8 Beneficial Roles and Uses of Microorganisms

1.8.1 Microbial Cell Factories

E. coli and *S. cerevisiae*, the best characterized prokaryote and eukaryote, respectively, have found tremendous biotechnological applications ranging from biochemical production to food, biopharmaceutical, biofuel, and enzyme production. *Corynebacterium glutamicum*, *E. coli*, and *S. cerevisiae* are well established as important industrial workhorses in biotechnology and pharmaceutical industries. *Ashbya gossypii* is used to produce about 4000 tons riboflavin per annum which represents about 50% of the global market volume (Becker and Wittmann 2015). Their "generally regarded as safe" status, high adaptability, non-fastidious growth requirements, and amenability to genetic manipulations make them the undoubted heroes of industrial microbiology and pharmaceutical biotechnology. A list of most commonly used microorganisms as microbial cell factories is provided in Table 1.3. The versatile microbial cell factories have offered a wide range of industrial products, viz., alcohols (mainly ethanol, propanol, butanol), organic acids (citric acid, lactic acid, succinic acid, itaconic acid), amino acids, insulin, cytokines, growth factors, diamines, vitamins, food-grade colors, β-carotene, lycopene, zeaxanthin, antibiotics, artemisinin, paclitaxel, anticancer agents, single-cell proteins, biofertilizers, industrial enzymes, etc. (Ferrer-Miralles and Villaverde 2013; Becker and Wittmann 2015). Microbial factories have been engineered to produce lipids from carbohydrate feedstocks for production of biofuels and oleochemicals. Similarly, biosynthesis of plant secondary metabolites in microbes provides an opportunity to significantly accelerate the drug discovery and development process (Demain and Sanchez 2009; Cragg and Newman 2013). Cyanobacteria like *Anabaena*, *Aphanocapsa*, *Calothrix*, *Microcystis*, *Nostoc*, *Oscillatoria*, and *Synechococcus* are potent source of hydrogen, alkanes, and alkenes (Mazard et al. 2016). *Arthrospira platensis* (*Spirulina*) is widely used for industrial production of nutraceuticals and vitamins (Demain and Sanchez 2009; Cragg and Newman 2013; Mazard et al. 2016).

1.8.2 Fermented Foods and Probiotics

Fermented foods are high-value foods and beverages produced by the controlled microbial metabolic activities. Fermented foods and beverages remain an integral part of the human diet since time prehistoric. The microorganisms most commonly employed in these processes are *S. cerevisiae*, *Acetobacter*, lactic acid bacteria (*Leuconostoc*, *Lactobacillus*, *Streptococcus*, *Lactococcus*), *Propionibacterium*

freudenreichii, *Bacillus*, and several filamentous fungi (Tamang et al. 2016; Marco et al. 2017). Some examples of fermented foods and beverages are alcoholic beverages, yogurt, cultured milk, cheeses, sauerkraut, pickles, bakery products, kimchi, sausages, etc. Microbial activities impart enhanced shelf life, organoleptic properties, nutritional value, and functional properties to the foods. The types, properties, microbes involved, and health benefits of fermented foods have been extensively reviewed by van Hylckama et al. (2011), Tamang et al. (2016), and Marco et al. (2017).

Probiotics are nonpathogenic bacteria or yeast that can survive the harsh environment of gastrointestinal tract to confer health benefits to the host when consumed in adequate amounts (O'Toole et al. 2017). WHO defines probiotics as "live microorganisms that, when consumed in adequate amounts, have a positive influence on the individual's health." The global probiotic market is projected to reach a turnover value of US $46.55 billion by 2020 (http://www.marketsandmarkets.com). The more commonly exploited strains/species among the lactobacilli and bifidobacteria have been accepted as having generally regarded as safe status (O'Toole et al. 2017). A list of various probiotic strains of microbes is given in Table 1.4. Health benefits of probiotics are well documented for prevention and treatment of acute gastroenteritis, antibiotic-associated diarrhea, necrotizing enterocolitis, allergies, infantile colic disease, *Helicobacter pylori* infection, cholesterolemia, lactose intolerance, irritable bowel syndrome, and certain types of cancer (Fontana et al. 2013; Doron and Snydman 2015; Chua et al. 2017).

1.8.3 Industrial Products from Microbes

Microbes are endowed with exceptional abilities to produce a plethora of primary and secondary metabolites under large-scale industrial or commercial production conditions. Various primary and secondary metabolites such as enzymes, alcohols, amino acids, organic acids, vitamins, antibiotics, pigments, flavors, biopharmaceuticals (insulin, cytokines), etc. are microbial products having global market value in billions of US dollars (Ferrer-Miralles and Villaverde 2013). In addition, microbial biomass, live microbial cells, and microbial biofuels constitute an important commercial market of microbial-synthesized products (Tables 1.3, 1.4 and 1.5). Extremophilic microorganisms represent untapped bio-resource having immense potential for biotechnological, chemical, and industrial applications. Thermophile and hyperthermophile organisms provide us with some of the most thermostable enzymes having multiple applications. These enzymes, called extremozymes, are extensively used in industries (food, paper, bakery, textile, leather, detergents, biofuels, pharmaceuticals, etc.) and biotechnology (Adrio and Demain 2014). Industrially important enzymes such as proteases, lipases, amylases, cellulases, xylanases, etc. are generally bio-sourced from thermophilic and alkalophilic microorganisms as described in Table 1.5. Specifically, the strains and species of genera *Bacillus*, *Clostridium*, *Thermus*, *Thermotoga*, *Pyrococcus*, *Thermococcus*, *Halobacillus*, *Halobacterium*, *Candida*, *Trichoderma*, *Halothermothrix*, etc. are potent producers of industrial enzymes (Ferrer-Miralles and Villaverde 2013; Adrio

Table 1.3 Microbial cell factories employed for the industrial productions of biopharmaceuticals, proteins, enzymes, organic acids, and many other bioproducts of industrial importance

Prokaryotic microbial cell factories	Eukaryotic microbial cell factories
Bacillus subtilis	*Aspergillus niger*
B. clausii	*A. oryzae*
B. amyloliquefaciens	*Ashbya gossypii*
B. megaterium	*Candida famata*
Brevibacterium lactofermentum	*Crypthecodinium cohnii*
Corynebacterium glutamicum	*Hansenula polymorpha*
Escherichia coli	*Mortierella alpina*
Pseudomonas fluorescens	*Penicillium chrysogenum*
Pseudomonas putida	*Pichia pastoris*
Ralstonia eutropha	*Saccharomyces cerevisiae*
Yarrowia lipolytica	*Schizosaccharomyces pombe*
	Trichoderma reesei

Table 1.4 Microbial species generally recognized for their beneficial role as probiotics (Compiled from Fontana et al. 2013; Doron and Snydman 2015; Chua et al. 2017)

Bacteria	Fungi
Escherichia coli Nissle 1917	*Saccharomyces cerevisiae*
Lactobacillus reuteri DSM 17938	*Saccharomyces boulardii* CNCM I-745
Lactobacillus rhamnosus GG	*Kluyveromyces* spp.
Lactobacillus acidophilus CL1285	*Torulaspora* spp.
Lactobacillus casei Shirota	*Pichia* spp.
Lactobacillus paracasei B21060	*Candida* spp.
Enterococcus faecium	
Bifidobacterium animalis subsp. *lactis* DN-173010	
Bifidobacterium longum subsp. *longum* 35624	
Streptococcus thermophilus	
Pediococcus pentosaceus	
Leuconostoc mesenteroides	
Bacillus subtilis	
Clostridium butyricum	

and Demain 2014; Krüger et al. 2017). Some details about the sources of microbial strains, genome databases, and other biotechnological and bioinformatic aspects are provided in Table 1.6.

1.8.4 Antibiotics and Biotherapeutics

Microorganisms are potent source of tremendously diverse bioactive metabolites. Serendipitous discovery of penicillin by Sir Alexander Fleming in 1929 is regarded as one of the most influential scientific breakthroughs of the last nine decades (Demain and Sanchez 2009). *Penicillium*, *E. coli*, *Aspergillus*, *Streptomyces*, *Bacillus*, *Cephalosporium*, *Trichoderma*, etc. are main producers of native or

Table 1.5 List of various industrial products, biopharmaceuticals, and other bioproducts derived from prokaryotic and eukaryotic microorganisms

Microbial products	Microbial source	Applications	References
Antibiotics (penicillins, tetracyclines, cephalosporins, quinolones, lincomycins, macrolides, sulfonamides, glycopeptides, aminoglycosides, carbapenems	*Streptomyces* spp., *Penicillium* spp., *Bacillus* spp., *Saccharopolyspora* sp., *Amycolatopsis* sp., *Micromonospora* sp., *Fusidium* sp. *Pseudomonas*	Treatment and control of bacterial, fungal, and protozoal infections in humans and animals	Demain and Sanchez (2009); Sarkar et al. (2017)
Anticancer chemotherapeutics (actinomycin D, bleomycin, doxorubicin, mithramycin, streptozotocin, epothilones, etc.)	*Streptoalloteichus hindustanus*, *Streptomyces peucetius*, *Sorangium cellulosum*	Treatment of cancers	Demain and Sanchez (2009); Cragg and Newman (2013)
Cholesterol-lowering drugs (lovastatin, simvastatin, mevastatin, pravastatin)	*Monascus ruber*, *Aspergillus terreus*, *Nocardia autotrophica*, *Penicillium citrinum*	In human health for reducing cholesterol and treating cancer	Lippi and Plebani (2017)
Biopharmaceuticals (recombinant human insulin, other hormones, cytokines, growth factors, interleukins, interferons)	*E. coli*, *Saccharomyces cerevisiae*	Diabetes, immunological disorders, cancers, growth disorders; as biosimilars and biobetters	Sanchez-Garcia et al. (2016); Bandyopadhyay et al. (2017)
Microbial enzymes (amylase, protease, lipase, pullulanase, cellulases, xylanase, glucoamylase, endoglucanase, endoxylanase, β-glucosidase, chitinase, pectinase)	*Bacillus licheniformis*, *B. stearothermophilus*, *B. amyloliquefaciens*, *Clostridium*, *Thermotoga*, *Methanopyrus*, *Pyrococcus*, *Thermococcus*, *Halobacterium*, *Halobacillus*, *Aspergillus*, *Penicillium*, *Streptomyces*	In food, baking, dairy, biopharmaceuticals, feed, agriculture, paper, pulp, leather, textile, detergents, biofuels, chemical, cosmetics, and bioremediation industries	Canganella and Wiegel (2014); Poli et al. (2017); Krüger et al. (2017)
Biofuels (biodiesel, bioethanol, isoprenoid, butanol, fatty acids, hydrogen)	*Clostridium acetobutylicum*, *Chlamydomonas reinhardtii*, *E. coli*, *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Synechococcus elongatus*, *Chlorella protothecoides*	An eco-friendly alternatives to conventional petroleum-based fuels	Kung et al. (2012); Show et al. (2017)
Microbial biomass protein (MBP)	*Spirulina* (*Arthrospira platensis* and *A. maxima*), *Saccharomyces cerevisiae*, *Chlorella*, *Dunaliella*, *Aspergillus*, *Chaetomium*, *Paecilomyces*, *Penicillium*, *Trichoderma*	Nutritional supplement for human; feed additive	Mazard et al. (2016)

(continued)

Table 1.5 (continued)

Microbial products	Microbial source	Applications	References
Single-cell oils [docosahexaenoic acid (DHA), polyunsaturated fatty acids (PUFAs), arachidonic acid (ARA), omega-3 fatty acids]	*Cryptococcus*, *Cunninghamella*, *Mortierella*, *Yarrowia*, *Schizochytrium*, *Thraustochytrium*, *Ulkenia*	Nutritional and biodiesel applications	Ochsenreither et al. (2016)
Vitamin B_{12}	*Pseudomonas denitrificans*, *Propionibacterium shermanii*, *Sinorhizobium meliloti*	In medicine and foods	Fang et al. (2017)
Microbial pigments/colorants (carotenoids, lutein, prodigiosin, lycopene)	*Chlorella protothecoides*, *Dunaliella salina*, *Haematococcus pluvialis*, *Murellopsis*, *Scenedesmus almeriensis*, *Monascus*, *Fusarium*, *Penicillium*, *Neurospora*, *Serratia marcescens*, *Blakeslea trispora*	In food, nutraceutical, health, feed, and cosmetics industries	Tuli et al. (2015); Show et al. (2017)

Table 1.6 Some prominent microbial culture collections, microbial data centers, sequence databases, microbiome projects, and nomenclature authorities

Organization/authority	Functions/related to	Website
American Type Culture Collection (ATCC)	Microbial culture collection	http://www.atcc.org/
Microbial Type Culture Collection (MTCC)	Microbial culture collection	http://www.imtech.ernet.in/mtcc/
National Collection of Dairy Cultures (NCDC)	Microbial culture collection	http://www.ndri.res.in/
CABI Genetic Resource Collection (IMI)	Microbial culture collection	http://www.cabi.org/
National Collection of Type Cultures (NCTC)	Microbial culture collection	www.phe-culturecollections.org.uk
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)	Microbial culture collection	http://www.dsmz.de/
National Agriculturally Important Microbial Culture Collection (NAIMCC)	Microbial culture collection	http://www.mgrportal.org.in
Collection de L'Institut Pasteur (CIP)	Microbial culture collection	http://www.crbip.pasteur.fr
Japan Collection of Microorganisms (JCM)	Microbial culture collection	http://jcm.brc.riken.jp/
Global Catalogue of Microorganisms (GCM)	Microbial information resource	http://gcm.wfcc.info/
List of Prokaryotic names with Standing in Nomenclature (LSPN)	Nomenclature authority and database	http://www.bacterio.net/
International Committee on Systematics of Prokaryotes (ICSP)	Nomenclature authority and database	http://www.the-icsp.org/
International Committee on Taxonomy of Viruses (ICTV)	Virus nomenclature authority	https://talk.ictvonline.org/
MycoBank	Fungal database & nomenclature	http://www.mycobank.org/
International Journal of Systematic and Evolutionary Microbiology (IJSEM)	Official journal of record for novel prokaryotic taxa and bacterial names	http://ijs.microbiologyresearch.org/content/journal/ijsem
Human Microbiome Project (HMP)	Microbiome project	http://www.commonfund.nih.gov/hmp
The Integrative Human Microbiome Project (iHMP)	Microbiome project	http://hmp2.org
The Earth Microbiome Project (EMP)	Microbiome project	http://www.earthmicrobiome.org/
Genomic Encyclopedia of Bacteria and Archaea (GEBA) initiative	Microbial genome sequencing	http://jgi.doe.gov/
GEBA-Microbial Dark Matter (GEBA-MDM) project	Microbial genome sequencing	http://jgi.doe.gov/

(continued)

Table 1.6 (continued)

Organization/authority	Functions/related to	Website
Tara Oceans (EMBL, French Center for Atomic Energy, CNRS, Council of Bretagne, French Ministry of Research)	Microbial genome sequencing	https://www.embl.de/tara-oceans/start/
Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP)	Microbial genome sequencing	http://marinemicroeukaryotes.org/
The Metagenomics and Metadesign of the Subways and Urban Biomes (MetaSUB) International Consortium	Microbial genome sequencing	http://metasub.org
Integrated Microbial Genomes & Microbiomes (IMG/M)	Genome sequence database	https://img.jgi.doe.gov/
Microbial Genome Database for Comparative Analysis (MBGD)	Completely sequenced microbial genome database	http://mbgd.genome.ad.jp/
Kyoto Encyclopedia of Genes and Genomes (KEGG)	Genome & metabolism database	http://www.genome.jp/kegg/
Ensembl Bacteria	Bacterial genome database	http://bacteria.ensembl.org/index.html
The Ribosomal Database Project (RDP)	Bacterial, archaeal, and fungal rRNA database	https://rdp.cme.msu.edu/index.jsp

Penicillin G

Streptozotocin

Cephalosporin

Vancomycin

Amphotericin B

Clavulanic acid

Bleomycin A_2

Streptomycin

Fig. 1.1 Some important microbial metabolites approved or in clinical trial phases for human and veterinary uses

recombinant biologically active compounds/drugs (Demain and Sanchez 2009; Cragg and Newman 2013; Table 1.5). In general, actinobacteria and filamentous fungi are a gold mine for production of novel antimicrobial, immunosuppressant, anticancer, and anticholesterogenic compounds (Fig. 1.1; Table 1.5). Recombinant

Tetracycline

Lovastatin

Actinomycin D

Doxorubicin

Atorvastatin

Paclitaxel

Epothilone A

Spinosyn A

Spinosyn D

Ivermectin B_{1a}

Ivermectin B_{1b}

Fig. 1.1 (continued)

Teixobactin

Caspofumgin

Cyclosporin A

Tacrolimus

Fig. 1.1 (continued)

human insulin, Humulin, was the first biopharmaceutical approved by FDA in 1982. In the last 70 years, more than 500 natural products have been approved by the US FDA for human use (Bandyopadhyay et al. 2017). Of these, about 400 products come under biopharmaceuticals such as recombinant proteins, peptides, monoclonal antibodies, and hormones. About 69% of these recombinant biopharmaceuticals are produced by *E. coli* and 5% in *S. cerevisiae* (5%) (Sanchez-Garcia et al. 2016). Immunosuppressive agents (cyclosporins), anticholesterol (lovastatin, mevastatin), anthelmintics, and antiparasitics (ivermectins) are some other pharmaceutical agents produced by microorganisms (Fig. 1.1). At the end of 2013, the total anti-infective drugs approved by FDA were 292, majority of which are either isolated from microbes or chemical derivatives of chemical moieties originally obtained from microbes (Kinch et al. 2014).

1.9 Conclusion and Future Directions

Microorganisms are nature's blessing to mankind. Their diversity, metabolic pathways, genetic repertoire, growth rate, and phenomenal adaptability are unmatched by any other group of living organisms on this planet. In the last few decades, impetus has been given to omics technologies, and microbiome studies to decipher the yet unknown and grossly underutilized metabolic potentials of the microbial world. With over 99.9% microbes yet to be cultured, it is almost certainly possible that enormous microbial species exists in nature which have tremendous power of synthesizing novel metabolites with improved functions and desired qualities. In the present scenario, the growing world population, extensive consumption of nonrenewable natural resources, emission of pollutants, environmental degradation, decreasing soil fertility, shrinking agricultural yields, emergence of plant pathogens, and compromised human health raise several red flags. Our real challenge is to find sustainable and environment-friendly solutions to these alarming issues. The human race looks forward to microorganisms for finding sustainable solutions of agriculture issues, environment pollution, metabolic diseases, public health, waste management, and reclamation of wasteland. Utilization of microorganisms as biofertilizers, biopesticides, biofungicides, next-generation probiotics, cell factories, nutraceuticals, live biotherapeutic products, microbial fuel cells, biofuels, biocement, etc. is considered as the most ethical approach toward self-sustaining and efficiently working ecosystems. Microbial potential for industrial and pharmaceutical purposes can be harnessed through the use of high-throughput screening, use of genetically engineered strains, improved fermentation technologies, and well-designed downstream processing methods. As our understanding about human microbiome is expanding, the focus has shifted to use microbial flora as signature of diseased conditions and manipulate the composition and abundance of same flora for the management and treatment of health conditions. Undoubtedly, the tiny yet powerful microorganisms can provide the rational, cost-effective, and sustainable solutions to ever-increasing needs of energy, food, medicine, and health of human populations.

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2 Bioherbicidal Concept: A Novel Strategy to Control Weeds

Vikas Kumar, Neeraj K. Aggarwal, and Anjali Malik

Abstract

Weeds have the potential to reduce the yield or quality of crops and produce a damaging effect on economic, social or conservation values. As peoples are more aware about environmental conservation, pressure is mounting for the development of effective and environment-friendly approaches to control weeds, on the scientists and active researchers in biological control field. Phytopathogenic microorganisms or microbial compounds, known as bioherbicides, for the biocontrol of weeds are most prominent alternative to minimize the hazardous chemicals/herbicides. Besides consistent research efforts and attempts in the area of bioherbicides throughout the globe, only 17 mycoherbicides have been registered worldwide. Advancement to develop mycoherbicides is relatively in suspended phase due to various restrictions like biological, economic and regulatory factors. Although only few bioherbicides are available in the market, biological control technology will be the leading approach to control unwanted plants. As the use of toxic herbicides is less desired because of their negative impact on crops, environment, ecosystem and human being, more funding needs to be applied to encourage researches for bioherbicides. In this review, we are concerned with various types of strategies, formulation of bioherbicides, steps in the development of mycoherbicide and constraints.

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Weeds · Bioherbicides · Phytopathogenic · Mycoherbicides · Formulations

2.1 Introduction

Of more than 300,000 species of plants known on the planet, barely 3000 are of monetary incentive to us (Burnside 1979). Among various aspects, weeds constitute one form which influences the productivity and sustainability of agricultural production. Weeds, i.e. wild plants that flourish where they are not needed, not exclusively, are a genuine disturbance in yards and greenery enclosures; they additionally stop up conduits, dislodge helpful plants from pastures and, in particular, develop among cultivated crops and contend with them for supplements, water and light (Agrios 2005). It is not surprising that losses caused by the weeds are encountered universally and crop yields are adversely affected. Infestation of weeds in general reduces crop yield by 31.5%, 22.7% in rabi crops (winter season) and 36.5% in *kharif* crops (summer season). Crop yields are lowered because weeds compete with crop plants for water, nutrients, light and space. Of the aggregate yearly loss of rural deliver from different nuisances in India, weeds generally represent 37%, insects for 29%, plant pathogens for 22% and remaining other pests for 12% (Yaduraju 2006). Li et al. (2003) revealed that out of around 30,000 types of generally disseminated weeds, 1800 species cause yield misfortune by around 9.7% of aggregate harvest creation consistently on the planet. Weeds cause $32 billion losses due to diminish 12% agricultural production (Chutia et al. 2007).

There are approximately 113 herbicide-resistant plant biotypes occurred in the United States out of total reported 307 weeds around the Universe. It has been reported that seven species of *Amaranthus* had become resistant to most of the herbicide available in the market (Heap 2006; Loretta et al. 2006). Continuous development of herbicide resistant in common agricultural weeds and owing to growing environment concerns (Heap 2006; Green et al. 1998) plant scientists and microbiologists have been prompted to find out alternative systems to manage weeds. Ideally, such frameworks ought to be a particular procedure against focused weeds without representing a risk to the earth and to the nontarget organisms. In the context, biological weed management practice, especially the use of bioherbicides, is an eco-friendly approach as the plant pathogenic fungi used as bioherbicides usually naturally where they are utilized, they are more selective in their mode of action and they are less toxic to people and animal than chemical herbicides. Numerous potential biological control agents of weeds have been observed to be hemibiotrophs. Hemibiotrophs have an underlying biotrophic stage took after by a necrotrophic stage (Bailey et al. 1992). This blend can bring about both generally high specificity and destructiveness (i.e. the level of pathogenicity).

Various factors like environment, economic and regulatory are some obstacles in the research and formation of successful mycoherbicides (Auld and Morin 1995). The future improvement of mycoherbicides is reliant on major information of

Table 2.1 Formulation details of mycoherbicides registered within two decades throughout the world (Aneja et al. 2013)

Trade name	Biological agent	Targeted weeds	Formulation type	Registration year
MycoTech™	*Chondrostereum purpureum*	Deciduous tree species	Paste	2004
Chontrol™ (EcoClear™)	*Chondrostereum purpureum*	Alder, aspen and other hardwoods	Spray emulsion	2004
Smolder[R]	*Alternaria destruens*	Dodder species	Conidial suspension	2008
Woad warrior	*Puccinia thlaspeos*	Dyer's woad (*Isatis tinctoria*) in farms, rangeland, waste areas and roadsides	Powder	2002
Sarritor	*Sclerotinia minor*	Dandelion (*Taraxacum officinale*) in lawns/turf	Granular	2007

natural collaborations at the creature and biological system level. Despite the interest in this area of weed control, there are few commercially developed bioherbicides. Besides the isolation of a huge naturally occurring pathogenic strains for conceivable use as mycoherbicides, yet just a little extent have been converted into biocontrol products. Several recent reviews have given an overview on different bioherbicide projects being directed around the world (Charudattan 2001; Aneja 2009; Ash 2010; Bailey et al. 2010; Aneja et al. 2013). At present, a sum of 17 mycoherbicides (8 in the United States, 4 in Canada, 2 in South Africa and 1 each in the Netherlands, Japan and China) have been enlisted over the world (Table 2.1).

2.2 Bioherbicide: Concepts and Approaches

Development of alternative and eco-friendly weed control strategies is needed to minimize perils coming about because of the presence of herbicide residues in the food chains and the ecosystem in general. Biological weed control has been proven to be major approach to achieve satisfactory control results and, meanwhile, reduce herbicide application to the minimum extent possible. Ideally, such systems should manage notorious weeds to the same extent as herbicides without posing a threat neither to the environment nor to nontarget species (Auld and Morin 1995; Boyette et al. 1996). Biocontrol strategy refers to the ponder utilization of natural enemies already present, to lessen the population of a target weed up to a coveted limit (Watson 1991). Exploitation of microbial-based formulation to prevent the infestation of weeds offers such an approach known simply as bioherbicide method (Fig. 2.1). In other words, bioherbicides are application of phytopathogenic organisms or their phytotoxic products for eco-friendly control of weeds in similar ways to commercial available herbicides (Boyetchko and Peng 2004). Most commonly fungi are used as biocontrol agents; hence the term mycoherbicide is often interchangeable used in these cases. Mycoherbicides have been referred to 'plant

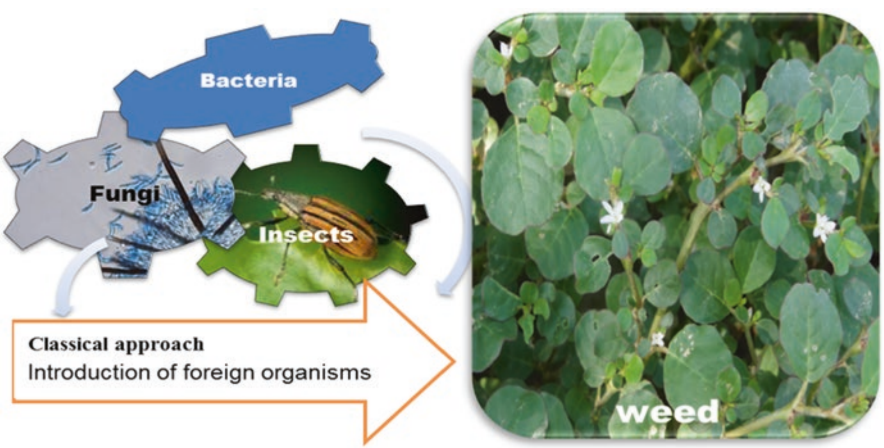

Fig. 2.1 Classical approach: Introduction of bacteria, fungi and insects to control weed

pathogenic fungi developed and used in the inundative strategy to control weeds in the way chemical herbicides are used' (TeBeest and Templeton 1985).

There are chiefly two strategies to execute the biocontrol by pathogens. The establishment of outside natural enemies, frequently called the '**classical strategy**', whereas second strategy named as '**augmentative**' or '**bioherbicide approach'**, where biocontrol agents are already present (native or introduced) in field and our concern, is to expand their population by mass rising up to desirable level. In epidemiological terms, such methodologies are likewise regularly portrayed as 'inoculative' and 'inundative strategy' (Charudattan and de Loach 1988; Hasan and Ayres 1990; Watson and Wymore 1990; Mueller-Schaerer and Frantzen 1996; Mueller-Schaerer and Scheepens 1997). Classical approach has been used since the historic time 1970s, when blackberry (*Rubus* spp.) in Chile (Oehrens 1977) and rush skeletonweed (*Chondrilla juncea*) in Australia were controlled by mean of two rust-causing fungi. But earlier and widely recognized example of success in classical biological control with fungi was management of rush skeleton weed by the rust fungus *Puccinia chondrillina* (Quimby 1982; Watson 1991; Hajek 2004). In the 1940s, research for mycoherbicide started to manage infestation caused by weeds with an earliest example of unsuccessful attempt to control the white form of the prickly pear (*Opuntia megacantha*) with use of *Fusarium oxysporum* in Hawaii (Fullaway 1954; Wilson 1969) and the successful application of *Acremonium diospyri* on persimmon (*Diospyros virginiana*) in the United States in the 1960s (Wilson 1965; Aneja et al. 2013).

Manipulated mycoherbicide strategy, considered as a third approach, has been proposed by Sands and Miller (1993). In contrast to previous approaches, lethal wide host range organisms are modified through genetic engineering to allow their secure and fruitful implementation to combat the constraints in release of mycoherbicides. It is possible they have rendered particular or given a synthetic reliance that ensures their stability or long-term survival. Introduction of molecular techniques may open the way to substantial scale corporate advancement and additionally also to bigger-scale public development of biocontrol agents.

2.3 Mechanism of Herbicide Resistance Among Weeds

Although herbicides have assumed a crucial part in enhancing crop yields and generally overall production efficiency, overdependence and redundant utilization of the herbicides belonging to the same can prompt the improvement of herbicide-resistant weed biotypes (Varshney and Prasad Babu 2008). For example, the continuous use of the broad-spectrum herbicide isoproturon in India had laid to the development of resistance in *Phalaris minor*, in 1993, thereby posing a serious threat to wheat cultivation in the country, especially in the northern states of Punjab and Haryana (Anonymous 2003). Four possible mechanisms of herbicide resistance in weeds include

1. Lack of rotation of the herbicides – if the same herbicides or different herbicides with the same mode of action are applied continuously on a weed, it will create selection pressure, thus allowing the resistant population to flourish.
2. Application of herbicides with high stable structure – this results in continued inhibition of susceptible biotype for a long period, thus providing extra benefits to resistant plants to grow.
3. Use of herbicides with highly specific mode of action – evolution of resistance against herbicides with highly specific mode of action will be quicker than against herbicides having multiple site of action.
4. Hypersensitivity of weeds to a particular herbicide (Tranel and Wright 2002).

2.4 Features Required for Probable Bioherbicide

Plants, similar to all organisms, are affected by various infections; however not all the infectious agents have the capacity to be selected as the candidate for potential biological control agents (Templeton 1992). Although in couple of cases, these illnesses have yielded phytopathogens which have been produced into commercial products in light of the fact that few plant pathogens are both deadly and sufficiently particular to be successful weed control agents (Sands and Miller 1993).

The qualities of plant pathogens that present them as attractive applicants have been widely checked by Daniel et al. (1973), Freeman et al. (1973), Freeman (1977), Templeton et al. (1979), and Aneja (1999). Desirable pathogens that could be utilized as appealing biological control agents have the accompanying features: (1) host range of the candidate should be very limited and must be have host specificity; (2) it should be easy to grow and produce infective units (e.g. spores, hyphae) on cost-effective media readily under suitable conditions; (3) it should be equipped for aggressive virulence bringing about successful control of targeted host; (4) they must have abnormal state of destructiveness; (5) they should have inefficient natural dispersion mechanisms; (6) product must be effectual under adequate diverse natural conditions; (7) the pathogen must be suitable of abundant production using traditional strategies such as liquid fermentation or solid state fermentation; (8) stability of the finalized product should be long-lasting; (9) storage (shelf life),

taking care of and techniques for utilization of formulation, must be compatible and accessible with present day agricultural system; (10) the biological control agent must be genetically steady; and (11) the agent gives speedy quick, perfect and effortless weed control.

2.5 Steps in Bioherbicide Development

Task in building up a mycoherbicide comprises of three stages or phases: discovery, advancement and deployment (Templeton 1992). The discovery stage includes the accumulation of diseased plant materials, screening and identification of the bio-control agents, demonstrating of Koch's hypothesizes, planning of the inoculum preparation on cheap manufactured culture media, large-scale production of spores and maintenance of the cultures in short-term and long-term storages. Developmental phase involves the assurance of ideal conditions for production of spores and for infection of the host and disease development, host range determination, and study of the mechanism how the pathogen kills the host. The development phase involves close joint effort between non-industrial and industrial sectors through the proper plan, application innovation, field assessment at small and large scale, acquiring enrolment of the item and lastly advertising phases of commercialization process (i.e. production of formulation on mass scale, promoting and dissemination) of a new bioherbicide product (Watson 1989).

2.6 Formulation of Mycoherbicide

Formulation is the crucial issue for inoculants containing a powerful biological control agent and can decide the achievement or failure of a mycoherbicide. A mycoherbicide formulation involves the mixing of the active ingredient, i.e. the fungal active units, with an adjuvant that may be carrier or solvent, to produce a form which can be effectively distributed on the target weeds (Boyette et al. 1991; Rhodes 1993). The formulation of a bioherbicides is a tool with which to store, apply and reduce the environmental dependency of the agent, these being major challenges in bioherbicide research (Green et al. 1998). Bioherbicidal Formulation may contain the viable agent in either inactive or metabolically active form (e.g. in the wake of drying spores and/or mycelia), has tendency to have longer life span of usability, is less demanding to pack and is more tolerant to ecological burdens, for example, temperature and relative humidity fluctuations and extremes. Alternatively, the formulations which contain the agent in metabolically active states have shorter shelf lives, less tolerant to environmental stresses, and require particular bundling to enable gaseous and moisture exchange (Paau 1988). The type of formulation used for a bioherbicide relies on the sort and method of the activity of the pathogen and accessible application innovation.

The formulation technology developed for one agent is not necessarily suitable for another. In most formulations, various agrochemicals like solvents, surfactants and

moisture retaining strategies are used which may be harmful for pathogenic organism (active ingredient) and limit the utilization of product (Connick Jr et al. 1991).

Majority of the bioherbicide formulations are based on the principle of maintaining viability of the agent in storage and educing dew period requirements. In general, bioherbicide formulations fall into two categories: liquid and solid formulations (Green et al. 1998).

2.6.1 The Liquid Formulations

The liquid formulations are used as post-emergence sprays that include aqueous-, oil- or polymer-based products to encourage leaf and stem diseases of targeted weeds. Water may be one of the bioherbicide delivery systems that contains the active propagules of the biocontrol agent formulated as a sprayable solution in water (Boyette et al. 1991; Auld 1992; Egley and Boyette 1995; Klein et al. 1995).

Adjuvants are the important compounds of any formulation that serve three main purposes: (1) they help or encourage the activity of the active ingredient, (2) enhance the qualities of the formulated product amid application and (3) hold the physical uprightness and stability of the formulation during the application (Foy 1989; Boyette et al. 1991; Womack and Burge 1993). Utilization of adjuvants in formulation increases the biocontrol efficacy and stability of the main active ingredient (Foy 1989).

Of the various compounds like surfactants, stickers, inert carriers, antifreezing compounds, humectants, sunscreening agents, anti-evaporation agent and micronutrients, the surfactants have been used widely in formulations of bioherbicides. These are involved in altering the wetting and spreading properties of the formulation (Prasad 1993, 1994). Simple sugars, proteins, pectins and pectinase, xanthan, gum and salts and extracts have also been used as adjuvants in several formulations for utilization by the biocontrol agent to enhance bioherbicide performance (Green et al. 1998). Use of adjuvants in the formulation of herbicides sometimes causes up to 100% mortality of target weed in 48 hours (Winder and Watson 1994). Yang and Jong (1995) prepared an inverted emulsion formulation of *Myrothecium verrucaria* by blending and watery spore suspension with oil (1:1 v/v), where just oil emulsion carrier killed the seven different weeds.

2.6.2 Solid or Granular Formulations

Solid or granular formulations of bioherbicides made up of grains, peat, charcoal, clay, vermiculite, alginate, bagasse, mineral oil or filter mud as carriers. For the preparation of solid formulation, the fungal propagules are initially produced through fermentation followed by mixing with the carrier material to form the final formulated products (Mortensen 1988). These formulations are better suited for the pathogens that infect weeds at or below the soil line and applied in pre-emergence applications, attacking the emerging weed seedlings as they come out from the soil

(Connick Jr 1988; Boyette et al. 1991). Since granular formulations contain dried inoculum, they have a longer life span than liquid formulations, which is an important criterion for the commercialization of a mycoherbicide (Auld 1992). Granular formulations also allow controlled release or growth of the organisms from the formulation (Rhodes 1993).

Alginate has been utilized in several granular bioherbicide formulations to form biodegradable pellets in which fungi can be readily entrapped. For example, it has been used to formulate *Alternaria cassia*, *A. macrospora*, *Fusarium lateritium*, *Colletotrichum malvarum*, *Phyllosticta* sp. (Connick Jr 1988; Walker and Connick Jr 1983), *Fusarium solani* (Weidmann 1988) and *Cercospora kikuchii* (Boyette and Walker 1985). The performance of alginate bioherbicide formulations is increased by the addition of nutritional amendments in several cases (Green et al. 1998).

2.7 Constraints in Bioherbicide Development

The pace of advancement in the field of commercialization of mycoherbicides is still moderate on account of a variety of science, financial and administrative imperatives. To beat these biological restrictions mainly pathogenicity, environmental stability, creating adequate numbers of spores to be economically reasonable, host range, ecological conditions, dew requirement of the pathogen and geographic biotypes of the weeds, the utilization of fungal biotechnology by molecular techniques like protoplast fusion, advancement of the fermenters, application techniques and modification of the carriers to the inoculum is being currently pursued in order to achieve the goal of advancement in myoherbicides and prepare useable products. Technological limitations like mass production and formulation have often created obstacles in bioherbicide advancements because these skills are outside the usual domains of weed scientists and plant pathologists. So, extensive research efforts are required to develop sufficiently cost-effective formulation (Boyette et al. 1991; Quimby et al. 1999). The opportunity expenses of formulation and fermentation specialists are relatively high, and major multinational organizations have been hesitant to relegate a lot of time for bioherbicide improvement to these researchers (Auld et al. 2003).

One reason behind this is that there is extensive writing in regard to simple distinguishing of specific pathogens for certain weed species which have potential for improvement yet moderately few papers managing large-scale manufacturing, plan, time span of usability and application (Auld and Morin 1995). Issues related with these elements are among a few that may oblige bioherbicide improvement. It has been proposed that an answer could originate from genetically engineering hypervirulence genes into weed-specific pathogens, where the pathogens are kept to avoid extinction by preservation of inoculum in the laboratory (Gressel et al. 2002). A great part of the discourse has focused on ordinary application procedures, but the future may well be extraordinary, and it is conceivable that the accomplishment of BCAs will be needy upon such innovative approaches. The successful deployment

of fungal BCAs relies upon close cooperation between all invested individuals. This incorporates researchers developing BCAs, makers who will deliver the agent, growers who wish to utilize the BCAs and government offices who frequently finance the research.

There is unmistakably a need to comprehend the biochemical and physiological parts of pathogenesis by the chosen fungal BCA with the goal that week links among host defence can be exploited.

2.8 Successful Bioherbicides

There is a long record of research on microbial biocontrol agents, and it is not generally valued that getting an active isolate is just the start of a progression of exercises fundamental for actualizing the utilization of another mycoherbicide (O'Connell and Zoschke 1996). There are various vital issues to be considered, including large-scale manufacturing, delivery systems and 'research center to field' studies and methodologies for utilization, enrolment and commercialization (Bateman 2001).

The quantity of research provides details regarding bioherbicide look into has expanded colossally since the mid-1980s. Scope for the biological control of notorious weeds increased or expanded in the recent time. There is also huge increase in unregistered bioherbicides around the world during previous time. In the same manner, the numbers of US licenses issued for the bioherbicidal utilization and their innovation have expanded, maybe prognosticating an expanded dependence on bioherbicides later on (El-Sayed 2005).

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3 Endophytic Microorganisms as Bio-inoculants for Sustainable Agriculture

Pratibha Vyas

Abstract

A sustainable crop production is one of the major challenges for agriculture in the twenty-first century. A considerable burden has been imposed on the agriculture by the overuse of chemical fertilizers to meet the demands of rising population. Environmentally safe and cost-effective solutions are required, focusing on reduced use of chemical fertilizers and pesticides, for improving productivity and sustainability in agriculture. The microorganisms living inside the plant tissues without causing apparent harm termed as "endophytes" are potential source of various novel compounds enhancing the plant growth and eliminating plant pathogens, which can be utilized for sustainable agriculture. Endophytic microorganisms are not only the promising source of growth metabolites but also enable the plant to resist stress-like conditions. These organisms have the potential to produce plant growth-promoting metabolites including phytohormones; enzymes like ACC deaminase, reducing the levels of ethylene; organic acids aiding in phosphate solubilization; and siderophores, cellulases, and chitinases inhibiting the growth of phytopathogens. The application of endophytic microorganisms with multifarious activities and biocontrol mechanisms could be beneficial in reducing the use of chemical fertilizers and pesticides for sustainable agriculture in the fragile ecosystems.

Keywords

Endophytes · Plant growth promotion · Phosphate solubilization · Phytohormones · Siderophores

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3.1 Introduction

One of the major challenges for agriculture in the twenty-first century is the production of environmentally sound and sustainable crops. Large amount of fertilizers and pesticides are used for enhancing agricultural yield to fulfill the demands of ever-increasing population. Since this has placed a considerable burden on the agriculture, so ecologically safe alternatives are required to improving productivity and sustainability in agriculture. One of the options is the use of microorganisms, as they have huge potential, thereby reducing the consumption of chemical fertilizers (Adesemoye and Kloepper 2009).

Microorganisms, an important component of ecosystems on earth, play an important role through the nutrient cycling, decomposition, and energy flow. These microorganisms make intimate association with plants and help in promoting plant growth and productivity by providing adequate supply of nutrients. Endophytes, residing within plant tissues during their life cycle without causing any harm, show a mutualistic association with their host plants, wherein the host plant supply sufficient nutrients and habitation for endophytes. In return, the endophytes synthesize large number of compounds including plant hormones, enzymes, organic acids, siderophores, hydrogen cyanide, antibiotics, and antifungal metabolites that help to grow the plants in a better way.

Endophytic actinomycetes, bacteria, and fungi have received great attention in terms of their ability to produce large number of agriculturally important compounds (Zhao et al. 2011). These microorganisms have been isolated from various sources and have the ability to promote plant growth and improve soil fertility because of the production of plant growth-promoting compounds (Passari et al. 2015; Pageni et al. 2014; Atugala and Deshappriya, 2015). Microbial endophytes are known to solubilize insoluble phosphates and produce plant hormones including auxins, cytokinins and gibberellins, siderophores, ammonia, hydrogen cyanide, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Moreover, they also help the plants to tolerate different stress conditions and grow better. They are known to produce extracellular enzymes like cellulases, proteinase, lipases, and esterases. Amides and amines and also very common metabolites produced by endophytes which have been proved to be toxic to insects but not to mammals.

3.2 Plants and Associated Endophytes

Microbial endophytes including actinomycetes, bacteria, and fungi can be “obligate” or “facultative,” wherein the former depends upon their host for growth and survival and the latter can exist outside the host plants. The relationship between the endophytes and their host plant ranges from latent phytopathogenesis to mutualism (Strobel and Long 1998). Endophytes may enter the interior of the root through auxin-induced tumors, wounds, or lateral branching sites or by hydrolyzing wall-bound cellulose (Hallmann et al. 1997; Siciliano et al. 1998). From nearly 300,000

plant species in the globe, each one hosts several to hundreds of endophytes (Qin et al. 2011), creating an enormous biodiversity.

Several endophytes are usually associated with a single plant, and among them, at least one species shows host specificity. They are known to be associated with many plants and have been isolated from many medicinal plants including *Curcuma longa*, *Picrorhiza kurroa*, *Tinospora*, *Withania somnifera*, *Zingiber officinale*, etc. (Table 3.1).

Table 3.1 Association of microbial endophytes with plants

Endophyte	Example	Plant	References
Actinomycetes	*Microbispora*, *Streptomyces*, and *Streptosporangium*	*Zea mays*	Araújo et al. (2000)
	Streptomyces sp.	*Rhododendron*	Shimizu et al. (2000)
	Streptomyces sp.	*Zingiber officinale*	Taechowisan et al. (2003)
	Streptomyces griseofuscus	Rice	Tian et al. (2004)
	Streptomyces griseorubiginosus	Banana (*Musa acuminata*)	Cao et al. (2004)
	Streptomyces, *Streptosporangium*, and *Streptoverticillium*	*Musa acuminata*	Cao et al. (2005)
	Streptomyces	Tomato	Tan et al. (2006)
	Streptomyces and *Micromonospora*	Chinese cabbage	Lee et al. (2008)
	Streptomyces spiralis, *Actinoplanes campanulatus*, and *Micromonospora chalcea*	Cucumber	El-Tarabily et al. (2009)
	Saccharopolyspora flava, *Rhodococcus fascians*, *Mycobacterium monacense*, *Gordonia sputi*, *Streptomyces hainanensis*, *Blastococcus aggregatus*, *Polymorphospora rubra*, *Micromonospora peucetia*, *Kineosporia aurantiaca*	*Maytenus austroyunnanensis*, *Cercidiphyllum japonicum*, *Paris yunnanensis*, *Maytenus austroyunnanensis*, *Tripterygium wilfordii*, *Maytenus austroyunnanensis*	Qin et al. (2009)
	Streptomyces, *Streptosporangium*, *Microbispora*, *Streptoverticillium*, *Saccharomonospora* sp., and *Nocardia*	*Azadirachta indica*	Verma et al. (2009)
	Streptomyces, *Nonomuraea*, *Actinomadura*, *Pseudonocardia*, and *Nocardia*	*Aquilaria crassna Pierre* ex Lec (eaglewood)	Nimnoi et al. (2010)

(continued)

Table 3.1 (continued)

Endophyte	Example	Plant	References
	Impatiens chinensis, *Senecio declouxii*, *Potentilla discolor*, *Stellera* sp., *Juncus effusus*, *Vaccinium bracteatum*, *Rhizoma* sp.	*Streptomyces aurantiacus*, *S. griseocameus*, *S. viridis*, *S. albogrisedus*, *Micromonospora*, *Oerskovia*, *Nonomuraea*, *Promicromonospora*, *and Rhodococcus*	Zhao et al. (2011)
	Brevibacterium sp., *Microbacterium* sp., and *Leifsonia xyli*	*Mirabilis jalapa* and *Clerodendrum colebrookianum*	Passari et al. (2015)
	Microbispora sp., *Streptomyces* sp., and *Micromonospora* sp.	*Emblica officinalis* Gaertn	Gangwar et al. (2015)
	Streptomyces flavoviridis	*Ocimum basillicum*, *Withania somnifera*, and *Rauvolfia tetraphylla*	Waheeda and Shyam (2017)
Bacteria	*Pseudomonas putida*, *Bacillus pumilus*, *Aureobacterium saperdae*, *Burkholderia solanacearum*, and *Phyllobacterium rubiacearum*	Cotton	Chen et al. (1995)
	Cellulomonas, *Clavibacter*, *Curtobacterium*, *and Microbacterium*	*Glycine max*, *Sorghum bicolor*, *Triticum aestivum*, *Zea mays*	Zinniel et al. (2002)
	Different taxonomic groups	*Alyssum bertolonii*	Barzanti et al. (2007)
	Achromobacter xylosoxidans, *Alcaligenes sp.*,	Sunflower	Forchetti et al. (2007)
	Bacillus, *Pseudomonas*, *Brevibacterium*	*Prosopis strombulifer*	Sgroy et al. (2009)
	Bacillus thuringiensis, *B. amyloliquefaciens*	*Chelidonium majus*	Goryluk et al. (2009)
	Bacillus and Sphingopyxis	Strawberry	Dias et al. (2009)
	Enterobacter sp.	*Populus trichocarpa*	Taghavi et al. (2010)
	Actinobacteria, *Proteobacteria*, *Bacteroidetes*, *Firmicutes*	*Solanum nigrum* L.	Luo et al. (2011)
	Bacillus, *Staphylococcus*, *Delftia*, *Paenibacillus*, *Methylobacterium*, *Microbacterium*, *and Stenotrophomonas*	*Phaseolus vulgaris*	Costa et al. (2012)
	Pseudomonas sp., *Bacillus megaterium*, *B. licheniformis*, *B. pumilus*, *Acinetobacter calcoaceticus*, *Micrococcus luteus*, and *Paenibacillus* sp.	Plectranthus tenuiflorus	El-Deeb et al. (2013)

(continued)

Table 3.1 (continued)

Endophyte	Example	Plant	References
	Pseudomonas sp.	*Zingiber officinale*	Jasim et al. (2014)
	Alcaligenes, *Bacillus*, *Curtobacterium*, *Pseudomonas*, *Staphylococcus*	*Phyllostachys edulis*	Yuan et al. (2015)
	Exiguobacterium profundum	*Amaranthus spinosus*	Sharma and Roy (2015)
	Alcaligenes faecalis	*Withania somnifera*	Abdallah et al. (2016)
	Bacillus cereus, *Bacillus pumilus*, *Pseudomonas putida*, *Clavibacter michiganensis*	*Curcuma longa*	Kumar et al. (2016)
	Pseudomonas sp.	*Tinospora cordifolia*	Kaur et al. (2017); Vyas and Kaur (2017)
Fungi	*Trichoderma citrinoviride*,*Paecilomyces marquandii*, *Acremonium furcatum*, *Cylindrocarpon pauciseptatum*, and *Chaetomium globosum*	*Actinidia macrosperma*	Lu et al. (2011)
	Alternaria, *Colletotrichum*, *Aspergillus*, *Fusarium*, *Gliocladium*, and *Cunninghamella*	*Malus sieboldii*	Cai and Wang (2012)
	Alternaria, *Fusarium*,*Cladosporium*, *Phomopsis*,*Colletotrichum*,*Clonostachys*, *Cosmospora*, *Cryptosporiopsis*, *Cylindrocarpon*, *Didymella*, *Epulorhiza*, *Myrmecridium*, *Leptosphaeria*, *Pyrenochaeta*, *Paraconiothyrium*, *and Stephanonectria*, and *Epulorhiza*	*Holcoglossum rupestre* and *H. flavescens*	Tan et al. (2012)
	Aspergillus niger, *A. flavus*, A. *nidulans*, *Penicillium chrysogenum*, *P. citrinum*, *Phoma*, *Rhizopus*, *Colletotrichum*, *Cladosporium*, and *Curvularia*	*Cannabis sativa*	Gautam et al. (2013)
	Chaetomium, *Alternaria*, *Cercophora*, *Fusarium*, *Hypoxylon*, *Nigrospora*, *Cladosporium*, *Thielavia*, *Schizophyllum*, *Gibberella*	*Cannabis sativa*, *Cedrus deodara*, *Pinus roxburghii*, *Picrorhiza kurroa*, *Withania somnifera*, *Abies pindrow*	Qadri et al. (2013)

(continued)

Table 3.1 (continued)

Endophyte	Example	Plant	References
	Alternaria tenuissima, *Aspergillus fumigatus*, *A. repens* *A. japonicus*, *A. niger*, *Fusarium solani*, *F. semitectum*, *Curvularia pallescens*, *Phoma hedericola*, *Drechslera australien*,	*Ricinus communis*	Sandhu et al. (2014)
	Pestalotiopsis, *Phomopsis*, *Aspergillus*, *Xylaria*, *Nectria*, *Penicillium*, and *Fusarium*	*Myrcia guianensis*	dos Banhos et al. (2014)
	Nigrospora, *Fusarium* sp.	*Crescentia cujete*	Prabukumar et al.2015
	Ramichloridium cerophilum	Chinese cabbage	Xie et al. (2016)

Different methods have been employed to isolate endophytic microorganisms from different plant parts. The methods involve the collection of plant parts including leaves, bark, roots, and stems depending upon the economic importance of the plant parts. The plant parts should be processed immediately or stored at 4 °C for 24 h. Processing involves the washing of plant parts under gentle flow of tap water to remove dust particles and surface microflora; sterilization using chemicals like ethanol, mercuric chloride, or sodium hypochlorite; and followed by washing with sterile distilled water three or four times to remove the disinfectants completely. The sterilized plant parts are then dried using tissue paper and cut into thin sections of 2–3 mm and kept onto the agar medium of choice. Other methods include the maceration of sterilized plant parts in normal saline or phosphate buffer saline, dilution of the suspension, and spreading on to appropriate agar medium. The colonies appearing around the plant parts are purified on the same medium and stocked in agar slants or glycerol for further studies.

3.3 Endophytes in Sustainable Agriculture

Microbial endophytes play an important role in sustainable agriculture and enhance plant growth and productivity by various mechanisms (Fig. 3.1). They also provide protection to plants because of their ability to produce large number of antimicrobial compounds and metabolites. They increase seedling emergence, plant establishment under unfavorable conditions, and plant growth. In addition, they also have the ability to degrade xenobiotics and organic compounds and resist heavy metals or antimicrobials, which may have arrived from their exposure to diverse compounds in the plants or soils. Endophytes including actinomycetes, bacteria, and fungi have been demonstrated to enhance plant growth and provide resistance to drought stress and tolerance to inappropriate soil conditions (Swarthout et al. 2009; Taurian et al. 2010).

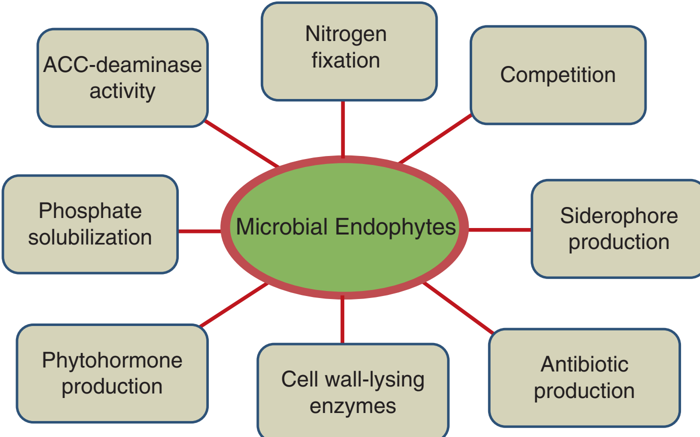

Fig. 3.1 Role of microbial endophytes in sustainable agriculture

3.3.1 Actinomycetes

Actinomycetes, gram-positive filamentous bacteria, in the domain bacteria are widely distributed in terrestrial and aquatic ecosystems (Ludwig and Klenk 2005). They help in decomposing complex materials including dead animals, plants, algae, and fungi and recycle nutrients, resulting in humus formation, thereby increasing the fertility of soils (Sharma, 2014). They have been reported to produce auxins, siderophores, and ammonia and also showed phosphate solubilization (Table 3.2). In addition, different genera have shown antagonism against phytopathogens and plant growth promotion. Endophytic *Streptomyces* spp. isolated from plants growing in Algerian Sahara showed IAA production (Goudjal et al. 2013). IAA production was affected by incubation period, pH, temperature, and tryptophan concentration. Highest IAA production of 127 μg/mL was observed in yeast extract-tryptone broth with 5 mgL-tryptophan/ml incubated for 5 days at pH 7, 30 °C and 200 rpm. Similarly, *Streptomyces*, *Nocardia*, *Nocardiopsis*, *Spirillospora*, *Microbispora*, and *Micromonospora* isolated from *Citrus reticulata* have shown IAA production ranging from 1.4 to 140 μg/ml (Shutsrirung et al. 2013).

Plant health is affected by pathogenic microorganisms which are a major threat to crop production. Several fungal species are plant pathogens causing major economic losses annually (Pennisi 2001). Actinomycetes are the largest group that has the ability to secrete large number of antibiotics inhibiting the growth of other organisms. Majority of the endophytic actinomycetes enhance plant growth by virtue of their ability to show antagonism against fungal pathogens (Table 3.2). In addition, they have been reported for phosphate solubilization and production of auxins, ammonia, and enzymes (Table 3.2). Siderophores are important compounds that help in the uptake of iron under iron-limited conditions. Several endophytic actinomycetes showing siderophore production have reduced the growth of fungal

Table 3.2 Endophytic actinomycetes in sustainable agriculture

Actinomycetes	Plant	Activity	References
Streptomyces sp.	*Rhododendron*	Antagonism against *Phytophthora cinnamomi* and *Pestalotiopsis sydowiana*	Shimizu et al. (2000)
Streptomyces sp.	*Zingiber officinale*	Antagonism against *Colletotrichum musae* and *Fusarium oxysporum*	Taechowisan et al. (2003)
Streptomyces griseofuscus	Rice	Antagonism against rice pathogens, rice blast disease, and sheath blast disease	Tian et al. (2004)
Streptomyces griseorubiginosus	Banana (*Musa acuminata*)	Antagonism against *Fusarium oxysporum* f. sp. *cubense*	Cao et al. (2004)
Streptomyces, *Streptosporangium*, and *Streptoverticillium*	*Musa acuminata*	Antagonism against *Fusarium oxysporum*	Cao et al. (2005)
Streptomyces sp.	Tomato	Antagonism against *Ralstonia solanacearum*	Tan et al. (2006)
Streptomyces and *Micromonospora*	Chinese cabbage	Biocontrol of *Plasmodiophora brassicae*	Lee et al. (2008)
Streptomyces spiralis, *Micromonospora chalcea*, and *Actinoplanes campanulatus*	Cucumber	Plant growth promotion, antagonism against *Pythium aphanidermatum*	El-Tarabily et al. (2009)
Actinomadura sp., *Microbispora* sp., *Micromonospora* sp., *Nocardia* sp., *Nonomuraea* sp.		IAA, siderophores, and antagonism against *Alternaria brassicicola*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Penicillium digitatum*, and *Sclerotium rolfsii*	Khamna et al. (2009)
Streptomyces, *Streptosporangium*, *Microbispora*, *Streptoverticillium*, *Saccharomonospora* sp., and *Nocardia*	*Azadirachta indica*	Antagonism against *Pythium* and *Phytophthora*	Verma et al. (2009)
Streptomyces, *Nonomuraea*, *Actinomadura*, *Pseudonocardia*, and *Nocardia*	Eaglewood	IAA, siderophores, ammonia, and protease	Nimnoi et al. (2010)
Streptomyces sp.	*Azadirachta indica* A. Juss	Siderophores and antagonism against *Alternaria alternata*	Verma et al. (2011)
Streptomyces enissocaecilis, *Streptomyces rochei, and Streptomyces plicatus*	Tomato	IAA	Goudjal et al. (2013)

(continued)

Table 3.2 (continued)

Actinomycetes	Plant	Activity	References
Streptomyces sp.	Rice (*Oryza sativa* L.)	Siderophore and plant growth promotion	Rungin et al. (2012)
Streptomyces sp.	*Taxus chinensis* and *Artemisia annua*	IAA	Lin and Xu (2013)
Streptomyces, *Nocardia*, *Micromonospora Microbispora*, *Nocardiopsis*, *and Spirillospora*	*Citrus reticulate* L.	IAA	Shutsrirung et al. (2013)
Streptomyces sp.		Phosphate solubilization, siderophores, ammonia, IAA, chitinase, amylase, cellulose, protease, and antagonism against fungal pathogens	Kaur et al. (2013)
Streptomyces, *Actinopolyspora*, *Saccharopolyspora*, and *Micromonospora*	*Aloe vera*, *Mentha arvensis*, and *Ocimum sanctum*	IAA, siderophores, and antagonisms against fungal pathogens	Gangwar et al. (2014)
Streptomyces cyaneofuscatus	Tomato	Plant growth promotion and antagonism against *Rhizoctonia solani*	Goudjal et al. (2014)
Streptomyces sp.	*Triticum aestivum*	Phosphate solubilization, phytase, chitinase, IAA production, siderophores, antifungal, plant growth promotion	Jog et al. (2014)
Streptomyces sp. and *Leifsonia xyli*	Medicinal plants	IAA production, siderophores, ammonia, chitinase, HCN, and antifungal activity	Passari et al. (2015)
Streptomyces sp., *Micromonospora* sp., *Microbispora* sp.	*Emblica officinalis* Gaertn	Phosphate solubilization, IAA production, siderophores, and antagonism against *Fusarium oxysporum*, *Rhizoctonia solani*, *Aspergillus niger*, *Alternaria brassicicola*, *and Phytophthora dresclea*	Gangwar et al. (2015)
Actinomycetes	*Syzygium cumini*	Proteinase and chitinase activity	Saini et al. (2016)
Streptomyces collinus, *S. diastaticus*, *S. fradiae*, *S. olivochromogenes*, *S. ossamyceticus*, and *S. griseus*	Different medicinal plants	Chitinase and antagonism against *Sclerotium rolfsii*	Singh and Gaur (2016)

phytopathogens and enhanced plant growth (Table 3.2). Endophytic Streptomyces sp. from rice enhanced the growth of rice and mung bean plants as compared to the plants inoculated with siderophore-deficient mutant treatments (Rungin et al. 2012).

3.3.2 Bacteria

Like endophytic actinomycetes, bacterial endophytes also use large number of mechanisms to promote plant growth. They have been known to enhance plant growth by various direct mechanisms like phosphate solubilization, fixation of nitrogen, and production of siderophores, ACC deaminase, and phytohormones (Glick et al. 2007). Indirect mechanisms are iron depletion, antibiotic production, fungal cell-wall degrading enzyme production, competition for sites, and induced systemic resistance (Glick et al. 2007; Sayyed and Chincholkar 2009). Endophytic bacteria *Bacillus pumilus*, *Pseudomonas putida*, *Burkholderia solanacearum*, *Aureobacterium saperdae*, and *Phyllobacterium rubiacearum* from cotton plants have shown antagonism *against Fusarium oxysporum* f. sp. *vasinfectum (Chen* et al. 1995*)*. In addition many endophytic bacteria have showed other traits of auxin production, *phosphate solubilization*, *nitrogen fixation*, *ammonia production*, *siderophore production*, and *ACC deaminase activity (*Table 3.3*). Many bacterial endophytes including Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Ideonella*, *Pseudomonas*, and *Sphingopyxis* enhanced *growth of different plants on inoculation (*Table 3.3*)*.

3.3.3 Fungi

Endophytic fungi are also essential components of sustainable agriculture as they can enhance growth and yield and improve plant fitness by providing biotic and abiotic stress tolerance. In addition, they also play a vital role in nitrogen and carbon cycling. Studies showed that fungal endophytes have important role in the biodegradation of host plant litter (Promputtha et al. 2010). They also release various secondary metabolites minimizing the effect of pathogens and provide defense to host plant against pathogenic microorganisms (Gao et al. 2010). Endophytic fungi *Aspergillus niger*, *Penicillium sclerotiorum*, *P. chrysogenum*, and *Fusarium oxysporum* isolated from *Camellia sinensis* growing in Assam, India, showed auxin production, phosphate solubilization, potassium solubilization, and zinc solubilization (Nath et al. 2015). Many fungal endophytes have shown their potential to be used in the production of cellulases, pectinases, proteases, and xylanases (Table 3.4). Chitin is an important constituent of the exoskeleton of insects and structural component of fungal cell wall. It is made up of linear homopolymer of β-1,4 linked N-acetylglucosamine. Chitinase enzyme obtained from endophytic fungi helps in degrading and recycling carbon and nitrogen from chitin, thereby playing an important role in maintaining the balance of the ecosystem (Table 3.4).

Table 3.3 Role of endophytic bacteria in sustainable agriculture

Bacterial Endophytes	Plant	Attribute	References
Herbaspirillum	Rice	*Nitrogen fixation*	Elbeltagy et al. (2000)
Herbaspirillum seropedicae	Rice	*Nitrogen fixation*	James et al. (2002)
Acinetobacter, *Enterobacter*, *Pantoea*, *Pseudomonas*, and *Ralstonia*	Soybean	*IAA*, *p-solubilization*, *nitrogen fixation*	Kuklinsky-Sobral et al. (2004)
Burkholderia sp.	*Vitis vinifera* L. cv. Chardonnay	*Plant growth promotion*	Compant et al. (2005)
Bacillus, *Burkholderia*, *Erwinia*, *and Pseudomonas*	*Paphiopedilum*	IAA	Tsavkelova et al. 2007
Achromobacter xylosoxidans, *Alcaligenes* sp., and *Bacillus pumilus*	Sunflower	Jasmonic acid, ABA	Forchetti et al. (2007)
Azospirillum spp.	Maize	IAA, nitrogen fixation	Roesch et al. (2007)
Bacteria	*Solanum nigrum*	ACC deaminase, IAA, phosphate solubilization	Long et al. (2008)
Acinetobacter, *Agrobacterium*, *Bacillus*, *Burkholderia*, *Pantoea*, and *Serratia*	Soybean	IAA, phosphate solubilization, nitrogen fixation	Li et al. (2008)
Serratia	Banana	Biocontrol against *Fusarium oxysporum*	Ting et al. (2008)
Burkholderia kururiensis	Rice	IAA	Mattos et al. (2008)
Pseudomonas, *Acinetobacter*, *Pantoea*, *Agrobacterium*, and *Aeromonas*	*Solanum nigrum*	ACC deaminase, IAA, phosphate solubilization	Long et al. (2008)
Achromobacter xylosoxidans	Wheat	IAA, phosphate solubilization, nitrogen fixation	Jha and Kumar (2009)
Bacillus, *Staphylococcus*, and *Klebsiella*	*Pachycereus pringlei* (Cactus)	Rock phosphate solubilization, nitrogen fixation, organic acids	Puente et al. (2009)
Bacillus, *Pseudomonas*, and *Brevibacterium*	*Prosopis strombulifer*	IAA, zeatin, gibberellic acid, abscisic acid, protease, ACC deaminase, phosphate solubilization, nitrogen fixation	Sgroy et al. (2009)
Bacillus and *Sphingopyxis*	Strawberry	IAA, phosphate solubilization, plant growth promotion	Dias et al. (2009)

(continued)

Table 3.3 (continued)

Bacterial Endophytes	Plant	Attribute	References
Pseudomonas putida, *Enterobacter* sp., *Serratia proteamaculans*, and *Stenotrophomonas maltophilia*	*Populus* spp.	IAA, ACC deaminase production, and growth promotion of plants	Taghavi et al. (2009)
Bacillus megaterium, *B. cereus*, *Micrococcus luteus*, *Lysinibacillus fusiformis*	Ginseng	IAA, phosphate solubilization, nitrogen fixation, siderophores	Vendan et al. 2010
Pantoea	*Arachis hypogaea*	Phosphate solubilization, siderophore, antagonism against fungal pathogens, and plant growth promotion	Taurian et al. (2010)
Pseudomonas sp.	*Alyssum serpyllifolium*	Plant growth promotion, Ni uptake	Ma et al. (2011)
Bacillus megaterium, *Pseudomonas putida*, and *Enterobacter sakazakii*	*Mammillaria fraileana*	Phosphate solubilization, nitrogen fixation	Lopez et al. (2011)
Ralstonia sp., *Pantoea agglomerans*, and *Pseudomonas thivervalensis*	*Brassica napus*	ACC deaminase, phosphate solubilization, IAA, metal resistance, siderophores	Zhang et al. (2011)
Achromobacter xylosoxidans, *Pseudomonas putida*, *Stenotrophomonas maltophilia*	*Amaranthus hybridus*, *Cucurbita maxima*, and *Solanum lycopersicum*	Phosphate solubilization, HCN, ammonia, antagonism against *Fusarium oxysporum*	Ngoma et al. (2013)
Bacillus	Avocado, black grapes	IAA, HCN, ammonia, protease, lipase	Prasad and Dagar (2014)
Azospirillum, *Burkholderia*, *Bradyrhizobium*, *Ideonella*, and *Pseudomonas acidovorax*	*Solanum tuberosum*	IAA, nitrogen fixation, antagonism against pathogens, plant growth promotion	Pageni et al. (2014)
Bacillus cereus, *B. pumilus*, *Pseudomonas putida*, and *Clavibacter michiganensis*	*Curcuma longa*	Phosphate solubilization, siderophores, antagonism against fungal pathogens *Fusarium solani* and *Alternaria alternata*	Kumar et al. (2016)
Pseudomonas sp.	*Tinospora cordifolia*	IAA, phosphate solubilization, siderophores, HCN, antagonism against fungal pathogens *Fusarium moniliforme*, *F. verticillioides*, plant growth promotion	Kaur et al. (2017); Vyas and Kaur (2017)

Table 3.4 Role of fungal endophytes in sustainable agriculture

Fungus	Plant	Activity	References
Williopsis saturnus	Maize	IAA	Nassar et al. (2005)
Trichoderma, *Nigrospora*, and *Curvularia*	*Rauwolfia serpentina*	Antagonism against *Fusarium oxysporum* and *Phytophthora* spp.	Li et al. (2000), Doley and Jha (2010)
Fusarium proliferatum	*Physalis alkekengi* var. *franchetii*	Gibberellins, plant growth promotion	Rim et al. (2005)
Acremonium zeae	Maize	Hemicellulase	Bischoff et al. (2009)
Hypoxylon sp.	*Persea indica*	Volatile organic compounds, antimicrobial activity against *Phytophthora cinnamomi*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Cercospora beticola*	Tomsheck et al. (2010)
Foliar endophytic fungi	*Pinus sylvestris* L. and *Rhododendron tomentosum* Harmaja	Ferricrocin siderophore	Kajula et al. (2010)
Aspergillus fumigatus, *Cladosporium sphaerospermum*, and *Talaromyces funiculosus*	Soybean	Bioactive gas	Hamayun et al. (2009)
Phoma glomerata and *Penicillium* sp.	Rice	Gibberellins, IAA, plant growth promotion	Waqas et al. (2012)
Aspergillus niger, *Penicillium sclerotiorum*, *P. chrysogenum*, and *Fusarium oxysporum*	*Camellia sinensis*	Auxin production, phosphate solubilization, potassium solubilization, and zinc solubilization	Nath et al. (2015)
Galactomyces geotrichum	*Trapa japonica*	IAA and biologically active gas	Waqas et al. (2014)
Fusarium tricinctum and *Alternaria alternata*	*Solanum nigrum*	IAA and plant growth promotion	Khan et al. (2015)
Trichoderma pseudokoningii	Tomato roots	Phosphate solubilization, IAA, siderophores, HCN, and ammonia	Chadha et al. (2015)
Penicillium chrysogenum, *Alternaria alternata*	*Asclepias sinaica*	IAA, ammonia, amylase, pectinase, cellulase, gelatinase, xylanase, tyrosinase, and plant growth promotion	Fouda et al. (2015)

(continued)

Table 3.4 (continued)

Fungus	Plant	Activity	References
Acremonium sclerotigenum inhabiting	*Terminalia bellerica Roxb*	Siderophore and also inhibits pathogenic microorganisms	Prathyusha et al. (2015)
Penicillium citrinum and *Aspergillus terreus*	*Helianthus annuus* L.	Plant growth and antagonism against *Sclerotium rolfsii*	Waqas et al. (2015)
Endophytic *Absidia* and *Cylindrocladium*	Rice	Plant growth promotion	Atugala and Deshappriya (2015)
Colletotrichum, *Lasiodiplodia*, and *Fusarium*		Siderophores	Aramsirirujiwet (2016)

Fungal endophytes have been reported to produce auxins, gibberellins, volatile compounds, siderophores, phosphate solubilization, and antagonism against fungal pathogens (Table 3.3). Endophytic *Piriformospora indica* has tremendous capacity to enhance growth of host plant through its root colonization (Waller et al. 2005; Varma et al. 2012). The molecular mechanisms by which the endophyte *P. indica* promotes growth and biomass production of various plant species have been studied (Lee et al. 2011). Prasad et al. (2013) reported enhancement of biomass and antioxidant activity in *Bacopa monnieri* when co-cultivated with *P. indica*. Endophytic fungi *Aspergillus fumigatus*, *Paecilomyces* sp., *Penicillium* sp., *Phoma glomerata*, *Chrysosporium pseudomerdarium*, and *Paecilomyces formosus* produced gibberellic acid and indole-3-acetic acid and also promoted shoot length, chlorophyll contents, and biomass of mutant and wild-type rice (Waqas et al. 2014a, b).

3.4 Conclusion and Future Prospects

Large use of chemical pesticides and fertilizers for increasing agriculture productivity has disturbed the ecological balance which has led to the buildup of pesticide resistance among pathogens. People are focusing on eco-friendly and safe approaches to increase agriculture productivity. Microbial endophytes are essential component of sustainable agriculture in view of their ability to produce large number of agriculturally important compounds and enhance plant growth. In recent years, research has also been focused on the use of genetically modified endophytes for improving plant yields and defensive properties. However, whatever is known about endophytes is not sufficient, and still some gaps exist in the studies carried out so far. Researchers are focusing on various genes helping particular microorganisms to invade plant tissues and provide a clue about their lifestyle. In the future, researchers would be able to engineer microbial endophytes for increasing their potential to be used as microbial inoculants, after fully understanding their function.

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Endophytes: A Gold Mine of Enzyme Inhibitors

4

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Abstract

Ever since the landmark discovery of paclitaxel from endophytic *Taxomyces andreanae*, plant endophytes have been the fountainheads of bioactive secondary metabolites with potential application in medicine, agriculture, and food industry. In the last two decades, lead molecules with antimicrobial, anticancer, antioxidant, and anti-inflammatory properties have been successfully discovered from endophytic microorganisms. Bioprospecting endophytes for enzyme inhibitors has been an important facet of endophytic research. Several enzyme inhibitors like altenusin, huperzine, camptothecin, and podophyllotoxin have been successfully isolated from endophytic microorganisms. The current chapter partially embodies the research progress on endophytic microorganisms for producing bioactive enzyme inhibitors and their possible use in pharmaceutical industries.

Keywords

Bioactive compounds · Endophytes · Enzyme inhibitors · Plant-microbe interaction

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4.1 Introduction

Development of resistance among pathogenic microorganisms, frequent appearance of life-threatening viruses, and tremendous increase in the incidences of communicable and noncommunicable diseases have drawn attention toward our inadequacy to manage these medical problems. This calls for an urgent need to exploit and utilize novel resources which could provide relief from the current situation (Strobel and Daisy 2003; Strobel et al. 2004). Natural products are metabolites or by-products of plant, animal, or microbial origin. Over the centuries, plants have been the cornerstone of natural products, but in the recent years, microbes associated with plants emerged as a key supplier of analogous and non-analogous bioactive metabolites with high therapeutic potential (Gouda et al. 2016; Meshram et al. 2016a). After the pathbreaking discovery of "Taxol" from *Taxomyces andreanae*, endophytes from various ecological niches of the world have been extensively exploited for obtaining bioactive metabolites having antimicrobial, anticancer, antiviral, and immunosuppressant activities (Zhao et al. 2011; Aly et al. 2011; Kusari et al. 2013). Endophytes are also found to inhibit specific enzymes and are commonly referred to as enzyme inhibitors. Since several diseases are associated with abnormal enzyme activities, the inhibitors bind to the active sites of the enzyme, thereby blocking the reaction that forms the basis of onset of disease. At present, several enzyme inhibitors like allopurinol, camptothecin, etoposides, febuxostat, lovastatins, mevastatin, and orlistat are available in the market (Baikar and Malpathak 2010; Gupta et al. 2015; Kapoor and Saxena 2014; Roy 2017). In the current chapter, we will discuss about the endophytes (including bacteria and fungi) as a novel bioresource of enzyme inhibitors and their possible application in management of several dreadful diseases.

4.2 Endophytes: A Potential Resource of Bioactive Metabolites

Endophytes comprise of an extremely diverse group of microorganisms that are ubiquitous in plants and maintain a symptomless and unobtrusive union with their hosts for at least a period of their life cycle (Stone et al. 2000; Saxena et al. 2015). The literal meaning of endophyte is "inside the plant" (Gr. endon, within; phyton, plants) (Schulz and Boyle 2005). Endophytic fungi are hyperdiverse and it is estimated that more than 1.5 million species may exist (Arnold et al. 2000). Fungal endophytes are more often encountered in comparison to bacterial endophytes. Once the endophyte enters the internal tissue of the host, they assume the latent phase for their entire life cycle or for an extended duration (Aly et al. 2011; Kaul et al. 2012). Their relationship with the host plant ranges from symbiotic, benign commensels, decomposers, to latent pathogens (Promputtha et al. 2007). During the alliance, none of the interacting partner is harmed, and the benefits obtained are solely dependent on the interacting partners. Thus, endophytism is a novel, cost-effective plant-microbe association driven by location and not by function (Kusari

et al. 2012). Endophytes produce a plethora of metabolites to cross talk with its host. These metabolites are produced in order to acquire nutrient and colonization inside the plant tissue and to provide defense against microbial infection (Borges et al. 2009). The bioactive metabolites obtained from endophytes majorly belong to the chemical class of alkaloids, cytochalasins, flavonoids, polyketides, steroids, and terpenoids (Porras-Alfaro and Bayman 2011). The metabolites produced by the endophytes have been found to exhibit various pharmacological properties, majority of which include antimicrobial, antineoplastic, antioxidant, anticancer, anti-inflammatory, antidiabetic, and antidepressant activities (Strobel and Daisy 2003; Strobel et al. 2004; Suryanarayanan et al. 2009; Kusari et al. 2013). Many biologically active metabolites like Taxol, camptothecin, oocydin, cytosporone, isopectacin, etc. have been successfully isolated from endophytic fungi possessing anticancer, antibacterial, antifungal, and antioxidant activities (Table 4.1) (Firakova et al. 2007; Zhao et al. 2011; Elsebai et al. 2014). Furthermore, endophytes were also found to produce various industrially and clinically important enzymes like amylase, cellulose, laccase, lipase, protease, etc. (Correa et al. 2014; Meshram et al. 2016a, b). Thus, endophytic microorganisms are rich source of biologically active metabolites possessing promising applications in agrochemical and pharmaceutical industries (Strobel and Daisy 2003; Kaul et al. 2012; Zilla et al. 2013; Zhang et al. 2015).

4.3 Enzyme Inhibitors

Enzymes are remarkable biological catalyst that efficiently and selectively catalyzes nearly all biochemical reactions inside a living system. Enzymes increase the rate of reaction by lowering the activation energy. Enzymes are highly specific in nature, and they bind only at the active sites of the substrate, ultimately converting them into products. However, due to some malfunctioning in the metabolic process, the level of enzyme activity is altered from the normal range, ultimately leading to serious metabolic disorders like Alzheimer's and Parkinson's disease, diabetes, and gout (Lehninger et al. 2005; Voet et al. 2013; Kapoor and Saxena 2014; Singh and Kaur 2015).

Agents that block or cease enzymatic reactions are known as enzyme inhibitor. These agents amend enzyme activity by combining in a way that influences the binding of substrate or its turnover number (Baikar and Malpathak 2010). Enzyme inhibitors are broadly classified into two categories: reversible and irreversible inhibitors. Reversible inhibitors are further subclassified into three categories: competitive, noncompetitive, and mixed inhibitors (Lehninger et al. 2005; Voet et al. 2013). Since enzymes carry out all the vital biological reactions, enzyme inhibitors are among the most important sought-after pharmaceutical agents. The current arsenal of pharmaceutical drugs largely comprised of enzyme inhibitors. Presently, almost all the therapies for AIDS are based on the suppression of certain vital enzymes (Roy 2017). At present, several enzyme inhibitors like 5-fluorouracil, cephalosporins, lovastatin, orlistat, penicillin, and ritonavir are available in the

Table 4.1 Bioactive secondary metabolites produced by endophytic fungi

S. no.	Bioactive compound	Endophytic fungi	Property	References
Anticancer agent				
1.1.	Paclitaxel	*Taxomyces andreanae*	Anticancer	Stierle et al. 1993
		Pestalotiopsis microspora		Strobel et al. 1996
1.2.	Camptothecin	*Entrophospora infrequens*	Anticancer	Puri et al. 2005
		Fusarium solani		Kusari et al. 2009a
1.3.	Podophyllotoxin	*Phialocephala fortinii*	Anticancer	Eyberger et al. 2006;
		Trametes hirsuta		Puri et al. 2006
4.	Vinblastine and Vincristine	*Fusarium oxysporum*	Anticancer	Kumar et al. 2013
5.	Torreyanic acid	*Pestalotiopsis microspora*	Anticancer	Lee et al. 1996
Antimicrobial agent				
6.	Cytosporones	*Cytospora* sp.	Antibacterial	Brady et al. 2000
7.	Brefeldin A	*Phoma medicaginis*	Antibacterial	Weber et al. 2004
8.	Sassafrins A–D	*Creosphaeria sassafras*	Antibacterial	Quang et al. 2005
9.	Pestaloside	*Pestalotiopsis microspora*	Antifungal	Lee et al. 1995a, b
10.	Cryptocandin A	*Cryptosporiopsis quercina*	Antifungal	Strobel et al. 1999
11.	Enfumafungin	*Hormonema* sp.	Anticandidal	Onishi et al. 2000
12.	Ambuic acid	*Pestalotiopsis microspora*	Antifungal	Li et al. 2001
Antiviral and antiparasitic agent				
13.	Pochonins A–F	*Pochonia chlamydosporia*	Antiviral and antiparasitic	Hellwig et al. 2003
14.	Pestalotheols A–D	*Pestalotiopsis theae*	Anti-HIV	Li et al. 2008
15.	Preussomerin EG1; palmarumycin CP_2, CP_{17}, and CP_{18}; and CJ-12,371	*Edenia* sp.	Antileishmanial	Martínez-Luis et al. 2008
Other important agents				
16.	Pestacin and isopestacin	*Pestalotiopsis microspora*	Antioxidant	Harper et al. 2003
17.	Subglutinol A	*Fusarium subglutinans*	Immunosuppressant	Lee et al. 1995a, b

(continued)

Table 4.1 (continued)

S. no.	Bioactive compound	Endophytic fungi	Property	References
18.	L-783,281	*Pseudomassaria* sp.	Insulin mimetic	Zhang et al. 1999
19.	Emodin	*Thielavia subthermophila*	Hypericin precursor	Kusari et al. 2008
1.20.	Diosgenin	*Cephalosporium* sp.	Cardiovascular therapy	Zhou et al. 2004
		Fusarium oxysporum	Estrogenic effect	Li et al. 2011

market, and hundreds of them are under clinical trials (Gupta et al. 2015; Drawz and Bonomo 2010). Most of the enzyme inhibitors reported to date are of microbial origin; hence in this section we will discuss about few important enzyme inhibitors isolated from endophytic microorganisms.

4.3.1 Angiotensin Converting-Enzyme (ACE) Inhibitors

Hypertension is the major risk factor that leads to various cardiovascular disorders, cirrhosis, and nephrosis. ACE is a vital component of renin-angiotensin system which maintains blood pressure in the body by regulating the volume of fluids. ACE converts inactive angiotensin I into physiologically active angiotensin II which causes an increase in blood pressure by contracting the blood vessels. Therefore, for the treatment of hypertension, it would be reasonable to administrate drug that inhibits ACE. Inhibitors of ACE bind to the active site of ACE enzyme, hence decreasing their action of narrowing the blood capillaries. Thus, ACE inhibitors are being widely used as hypertensive drugs. Several ACE inhibitors like benazepril, captopril, and ramipril are available for clinical use (Steven-Miles et al. 1995; Zhang et al. 2000; Coates 2003; Barbosa-Filho et al. 2006).

Endophytic *Cytospora* sp. isolated from living bark of *Betula alleghaniensis* produces three different phenolics named as cytosporin A (major), cytosporin B (minor), and cytosporin C (minor). These compounds bind to both angiotensin I and II at different levels with different specificities. Maximum inhibition of angiotensin II was shown by cytosporin A with an IC_{50} value of 1.5–3.0 μM. It also inhibited angiotensin I with an IC_{50} value of 25–30 μM. The other two cytosporins were better inhibitors of angiotensin II than angiotensin I. (Table 4.2, Fig. 4.1) (Steven-Miles et al. 1995).

Graphislactone A and botrallin produced by endophytic *Microsphaeropsis olivacea* exhibited moderate ACE inhibitory activity with an IC_{50} values of 8.1 and 6.1 μg/mL, respectively (Hormazabal et al. 2005). Further, *Pestalotiopsis* spp., isolated from *Terminalia arjuna* and *Terminalia chebula*, has also been reported to inhibit ACE with an inhibition greater than 60%. Out of 32 screened *Pestalotiopsis* spp., only 5 species showed ACE inhibition. From these five species, *Pestalotiopsis microspora* was the most potential one followed by *Pestalotiopsis theae* with an IC_{50}

Table 4.2 List of important enzyme inhibitors from endophytic fungi

Enzyme inhibitor	Enzyme	Source	Targeted disease	References
Cytosporin A	Angiotensin-converting enzyme	*Cytospora* sp.	Hypertension	Steven-Miles et al. 1995
Huperzine A	Acetylcholinesterase	*Shiraia* sp.	Alzheimer's disease, Parkinson's disease, Glaucoma	Zhu et al. 2010
Nectriapyrone	Monoamine oxidase	*Erythrina crista-galli*	Neurological, Psychiatric disorders	Weber et al. 2005
Aurovertin B–D	ATPase	*Calcarisporium arbuscula*	Cardiovascular disorders, Ulcers	Mao et al. 2015
Aurasperone A, rubrofusarin B	Xanthine oxidase	*Aspergillus niger*	Gout	Song et al. 2004
Polyhydroxy anthraquinones	Quorum sensing	*Penicillium restrictum*	Bacterial infections	Figueroa et al. 2014
Bipolarisenol	Urease	*Bipolaris sorokiniana*	Rheumatoid arthritis	Khan et al. 2015
Cytonic acids A–B	Protease	*Cytonaema* sp.	Viral infections	Guo et al. 2000
Solanapyrone A	DNA polymerase	*Alternaria solani*	Cancer, viral infections	Mizushina et al. 2001
Altenusin	Trypanothione reductase	*Alternaria* sp.	Trypanosomiasis	Cota et al. 2008
Radicicol	Heat shock protein 90 kD	*Chaetomium chi-versii*	Cancer	Turbyville et al. 2006
Epicocconigrones A	Histone deacetylases	*Epicoccum nigrum*	Cancer	El Amrani et al. 2014
Fusaristatin A	Topoisomerases I and II	*Fusarium* sp.	Cancer	Shiono et al. 2007
Corynesidone A	Aromatase	*Corynespora cassiicola*	Breast cancer	Chomcheon et al. 2009
Peptide	α-Amylase	*Aspergillus awamori*	Diabetes	Singh and Kaur 2015
Peptide	α-glucosidase	*Aspergillus awamori*	Diabetes	Singh and Kaur 2015
Lovastatin	HMG-CoA reductase	*Phomopsis vexans*	Cholesterol inhibitor	Parthasarathy and Sathiyabama 2015
Fustat	Lipase	*Fusarium incarnatum*	Obesity	Gupta et al. 2015 (Patent filing under process)

Fig. 4.1 Structures of important enzyme inhibitors from endophytic fungi. (Structures are taken from protologue publications and are redrawn using ChemDraw)

values that range from 21 to 37 μg/mL. These values were quite comparable to captopril (Tejesvi et al. 2008). Thus, it is clearly evident that endophytes are potential but scarcely studied candidates for ACE inhibitors. Hence, there exist immense opportunities to harness endophytic microflora as a novel bioresource of ACE inhibitors.

4.3.2 Acetylcholinesterase Inhibitors

Acetylcholinesterase (AChE) belongs to the family of serine hydrolyses that is primarily found at neuromuscular junctions and cholinergic brain synapses. AChE is majorly involved in termination of impulse transmission at cholinergic synapses by rapid breakdown of AChE into acetate and choline. This reaction is critical because it allows the cholinergic neuron to return back to its latent state after activation (Colovic et al. 2013). The inhibitors of AChE bind at the active site of the enzyme resulting in the accumulation of the acetylcholine at the synapses. Acetylcholinesterase inhibitors (AChEIs) are broadly classified as strong and weak inhibitors. The strong inhibitors comprise of organic phosphates and carbamates which are primarily used as nerve toxins, whereas the weak inhibitors have been employed in the treatment of Alzheimer's disease, autism, dementia, insomnia, and Parkinson's disease. The current arsenal of drugs involved in

treatment of these diseases includes galantamine, huperzine A, donepezil, and rivastigmine. The first two drugs are naturally obtained, whereas the latter ones are chemically synthesized. Even though AChEIs have been obtained from both chemical synthesis and natural resources including plants and microorganisms, the search for alternative avenues for isolating novel AChEIs is still going on (Rodrigues et al. 2005; Su et al. 2017).

The very first attempt to exploit endophytic fungi as a potential source of AChEIs was done by Rodrigues et al. (2005) where they have screened the culture filtrates obtained from the endophytic fungi isolated from Anacardiaceae, Apocynaceae, Leguminosae, and Palmae plant families. The maximum AChE inhibition recorded by these isolates was 43%. Endophytic fungal isolates like *Pestalotiopsis guepini*, *Phomopsis* sp., and *Guignardia mangiferae* displayed selective AChE inhibition, whereas *Chaetomium* and *Xylaria* spp. do not show any inhibitory activity (Rodrigues et al. 2005). Further, endophytic *Alternaria* spp. have been reported to exhibit AChE inhibitory activity. The chloroform extract of endophytic *Alternaria* sp. isolated from the *Ricinus communis* showed a strong AChE inhibitory activity with an IC_{50} value of 40 μg/mL (Singh et al. 2012). Similarly, endophytic *Alternaria alternata* isolated from *Catharanthus roseus* produces "altenuene" which exhibited 78% inhibition of AChE under in vitro conditions. The compound also possessed antioxidant and antilarval activity (Bhagat et al. 2016). Recently, endophytic fungus *Bipolaris sorokiniana* LK12 produces a radicinol derivative, "bipolarisenol," which significantly inhibited AChE with a low IC_{50} value of 67.23 ± 5.12 μg/mL (Khan et al. 2015).

Huperzia serrata is a traditional Chinese medicinal plant producing a lycopodium alkaloid huperzine A, which is a selective and reversible AChEI (Liu et al. 1986a, b). Huperzine A possesses better inhibitory activity than its counterparts donepezil and tacrine owing to its greater half-life, higher oral bioavailability, and lesser known side effects (Zhao and Tang 2002; Zangara 2003; Ma et al. 2007). Endophytic microorganisms possess a special property of synthesizing analogous compounds similar to their host (Saxena et al. 2015). Endophytic fungal isolate *Shiraia* sp. Slf14 associated with *Huperzia serrata* produced 327.8 μg/l of huperzine A which was higher than that from the previously reported endophytic isolates *Acremonium* sp., *Blastomyces* sp., and *Botrytis* sp. Furthermore, huperzine A from *Shiraia* sp. Slf14 exhibited dose-dependent AChE inhibitory activity. About 10 μg/ml of huperzine A from methanolic extract of endophytic fungus showed complete inhibition of AChE which was better than that of commercially available huperzine A under laboratory conditions (Table 4.2, Fig. 4.1) (Li et al. 2007; Ju et al. 2009; Zhu et al. 2010). Similarly, two endophytic *Penicillium* sp. L10Q37 and *Penicillium* sp. LQ2F02 isolated from *Huperzia serrata* produce several AChEIs. Ethyl acetate fraction of both the isolates showed 61 and 66% AChE inhibitory activity. Among the different compounds (S1–S10) produced by the two isolates, the lowest IC_{50} was exhibited by compound S5 (5.23 ± 0.28 μg/ml) under in vitro conditions (Wang et al. 2015). Apart from producing analogous compounds, several other bioactive metabolites were also isolated from *Huperzia serrata*. An endophytic fungal isolate

Aspergillus versicolor Y10 produces prenyl asteltoxin derivatives "avertoxins A–D" which also showed AChE inhibitory activity. Among them, avertoxin B (3) was the major compound showing AChE inhibitory activity with IC_{50} value of 14.9 μM (Wang et al. 2015). Thus, from the above reports, it looks apparent that endophytes are good candidates for the AChEIs. However, looking at the broad diversity of the endophytic microorganisms, various ecological niches around the world need to be exploited in a more rational and precise manner for recovering promising AChEIs with potential therapeutic application.

4.3.3 Monoamine Oxidase Inhibitor

Monoamine oxidase is an intramitochondrial enzyme that catalyzes the oxidative deamination of neurotransmitters such as dopamine, serotonin, and norepinephrine in the central nervous system leading to neurological and psychiatric disorders (Meyer et al. 2006). Low levels of these neurotransmitters lead to anxiety, depression, and schizophrenia (Domino and Khanna 1976). Inhibitors of monoamine oxidase obstruct the action of monoamine oxidase enzyme, thereby increasing the amount of neurotransmitters and thus providing relief from depression and anxiety (Tan et al. 2000). Presently, several monoamine oxidase inhibitors (MOI) including isocarboxazid, selegiline, phenelzine, rasagiline, and tranylcypromine are available in the market for treatment of neurodegenerative conditions. MOI are only used when other antidepressants have failed to work because they suffer from higher risk of drug interaction (Kennedy 1997; Weinreb et al. 2010; Wallach et al. 2017). Since, the currently available MOI also suffer from several drawbacks; the demand for new MOI with fewer side effects is highly desirable.

Weber et al. (2005) documented the production of nectriapyone from extract of *Phomopsis* species. The lead molecule was earlier reported to possess MAO inhibitory activity (Table 4.2, Fig. 4.1) (Lee et al. 1999). Similarly, hypericin is a naturally occurring antidepressant found in several species of *Hypericum perforatum.* Endophytic *Thielavia subthermophila* isolated from *H. perforatum* produces hypericin (Kusari et al. 2008). Metabolites like formamide and furansteroid, produced by endophytic *Talaromyces* sp. isolated from the bark of *Tripterygium wilfordii*, exhibited moderate MAO inhibitory activity (Zhao et al. 2016; Zhi et al. 2016). Further, mullein isolated from the culture broth of *Colletotrichum gloeosporioides* GT-7 exhibited monoamine oxidase inhibitory activity with an IC_{50} value of 8.93 ±0.34 μg/ml (Wei et al. 2016). Furthermore, deacetylisowortmins A and B isolated from an endophytic *Talaromyces wortmannii* LGT-4 also displayed weak monoamine oxidase inhibitory activity (Fu et al. 2016). MOI from endophytic microorganisms are a nascent area with very scanty and preliminary data. However, the available reports suggest that endophytes are prospective microorganisms for isolation of new MOI.

4.3.4 Adenosine Triphosphatase (ATPase) Inhibitors

ATPase is a broad class of enzymes that catalyze the hydrolysis of adenosine triphosphate into adenosine diphosphate and a free phosphate ion, liberating energy which is used for carrying out major biochemical reactions in the body (Chene 2002). ATPase is involved in vital cellular functions like DNA replication and synthesis (Lee and Bell 2000), protein folding and transport (Ranson et al. 1998), and transmembrane ion exchange (Hirokawa et al. 1998; Nishi and Forgac 2002). Several ATPase inhibitors like monastrol, digoxin, benzimidazoles, brefeldin A, sodium orthovanadate, and oligomycin A are already present in the market which play significant role in treatment of diseases like cancer, cardiovascular disorders, gastric disorders, and infections (Chene 2002; Cochran and Gilbert 2005; Sato et al. 2012). This is the reason why ATPase inhibitors hold a special position in pharmacopeia.

Digoxin is a plant glycoside produced by *Digitalis lanata*. The glycosides from this plant possess cardiotonic properties. Kaul et al. (2012) screened 32 endophytic fungal isolates isolated from *Digitalis lanata* and found that 5 isolates showed digoxin production under in vitro conditions. Aurovertin is a fungal polyketide that inhibits ATP synthase. Endophytic *Calcarisporium arbuscula* produces aurovertin B and D which are presently under clinical trial for human use (Table 4.2, Fig. 4.1) (Mao et al. 2015). Aurovertin-type polyketides T and U showed potential cytotoxic activity against triple negative breast cancer (Zhao et al. 2016). Similarly, oligomycin is also an inhibitor of ATP synthase. Neomaclafungins A−I produced by marine-derived actinomycete exhibited strong antifungal activity against *Trichophyton mentagrophytes* with a MIC value between 1 and 3 μg/mL (Sato et al. 2012).

Brefeldin A is a lactone antibiotic and ATPase inhibitor. Endophytic *Cladosporium* sp. isolated from *Quercus variabilis* exhibited brefeldin A production (Wang et al. 2007). Further, endophytic *Paecilomyces* sp. and *Aspergillus clavatus* isolated from *Taxus mairei* and *Torreya grandis* produced brefeldin A which exhibited cytotoxic activity against human tumor cell lines including HL60, KB, Hela, SPC-A-1, and MCF-7 (Wang et al. 2002). Similarly, the ethyl acetate extract of endophytic *Penicillium janthinellum* Yuan-27 also exhibited brefeldin A production which was active against human cancer cell lines like MKN45, LOVO, A549, MDA-MB-435, HepG2, and HL-60 with an IC_{50} value of <0.12 μg/ml (Zheng et al. 2013).

4.3.5 Xanthine Oxidase Inhibitors

Purine catabolism is an enzymatically driven metabolic pathway yielding uric acid as its final product. Xanthine oxidase is a key enzyme of this pathway which catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid. Any impairment in this finely regulated mechanism leads to a condition known as hyperuricemia. Hyperuricemia can be defined as a biochemical abnormality caused due

to high serum urate levels attributed due to overproduction (> 6–7 mg/dl) or under-excretion of uric acid. Further, gout is a common metabolic disorder characterized by chronic hyperuricemia and clinically manifested by unbearable pain in the joints (Lehninger et al. 2005; Voet et al. 2013). Over 4.62 million people across the globe suffer from hyperuricemia or gout. Gout can be prevented by antihyperuricemic therapy involving uricosuric drugs or xanthine oxidase inhibitors (XOI). Among the abovementioned strategies, XOI are more preferred ones, owing to their lesser side effects and interventions with purine catabolism (Kapoor and Saxena 2014, 2016). XOI are of two kinds: purine analogue and non-purine analogue. Purine analogue includes allopurinol, oxypurinol, and tisopurine, whereas non-purine analogue includes febuxostat and inositols (Lehninger et al. 2005). Presently, only allopurinol and febuxostat are clinically approved as XOI. However, there is an increasing demand of new XOI due to side effects encountered by the current drugs (Gu 2009).

Several endophytic fungi have been reported to exhibit XOI activity. Endophytic *Fusarium* sp. IFB-121 isolated from *Quercus variabilis* produced two compounds: a known cerebroside and a fusaruside exhibiting xanthine oxidase inhibitory activity with an IC_{50} values of 55.5 ± 1.8 μM and 43.8 ± 3.6 μM, respectively (Shu et al. 2004). Aurasperone A and rubrofusarin B obtained after fractionation of organic extract of endophytic *Aspergillus niger* IFB-E003 showed profound xanthine oxidase inhibitory activity with the IC_{50} values ranging from 10.9 to 37.7 μmol/l (Table 4.2, Fig. 4.1). The compounds also possessed broad spectrum antimicrobial and anticancer activity (Song et al. 2004). Similarly, endophytic *Chaetomium* sp. isolated from the *Nerium oleander* exhibited xanthine oxidase inhibitory activity with an IC_{50} value of 109.8 μg/mL. The same fungus also showed strong antioxidant activity (Huang et al. 2007). Lumichrome, produced in the liquid culture of endophytic *Myrothecium roridum* IFB-E012, displayed inhibition of xanthine oxidase with an IC_{50} value of 60.32 ± 0.48 μmol/l. The compound also displayed strong cytotoxic activity against human tumor cell line nasopharyngeal epidermoid KB (Li et al. 2009). Similarly, “alternariol” produced by endophytic *Alternaria brassicicola* ML-PO8 exhibited a comparable xanthine oxidase inhibitory activity with an IC_{50} value of 15.5 μM (Gu 2009). Two non-purine XOI were isolated from the culture filtrate of endophytic *Lasiodiplodia pseudotheobromae* and *Muscodor darjeelingensis respectively*. The IC_{50} values of XOI from the two endophytes were 0.61 and 0.54 μg/ml, respectively, which were much lower than allopurinol but were higher than that of febuxostat under in vitro conditions. Furthermore, both the isolates showed 84–88% reduction in the uric acid production which is comparable with the commercially available drugs under laboratory conditions (Kapoor and Saxena 2014, 2016). Recently, silver nanoparticles synthesized from the extract of endophytic *Penicillium* sp. also displayed the ability to strongly inhibit xanthine oxidase (IC_{50}: 92.65± 1.81 μg/mL). Further, the fungus also showed strong antibacterial, antioxidant, and antilipoxygenase activity (Govindappa et al. 2016). The published reports suggest that endophytes can be taken into account for the development of novel XOI.

4.3.6 Quorum Sensing Inhibitors

Quorum sensing is a process of communication between the bacterial cells that involves the production, detection, and response to an extracellular signaling molecule known as autoinducers. These autoinducers increase in concentration as a function of cell density (Rutherford and Bassler 2012). At low cell densities, bacteria behave as unicellular organisms; however they shift their behavior to multicellular type following stimuli that their cell densities have reached a threshold level (Kalia 2013). The intensity of communication signal reflects the population of bacterial cells in a particular environment, and hence the level of signal ensures that density of bacterial cells is enough to make behavioral changes which are termed as "quorate" (Hentzer and Givskov 2003). This mechanism enables bacteria to overpower human defense system and cause various diseases. To control the virulence of particular pathogenic bacterial species, these communication channels between the bacterial cells need to be ceased. Quorum sensing inhibitors showed promising effect as an alternative to antibiotics, and this is the reason why several have been largely studied from synthetic and natural resources (Defoirdt et al. 2013).

Quorum sensing mechanism can be measured by studying the ability to suppress violacein production by the sensor stain *Chromobacterium violaceum* (Rajesh and Rai 2014). Ma et al. (2013) screened over 1100 endophytic isolates isolated from tobacco leaf for their quorum sensing inhibitory activity. Out of 1177, only 168 isolates showed strong quorum quenching ability. Among them, *Lysinibacillus fusiformis*, *Pseudomonas geniculata*, *Serratia marcescens*, and *Bacillus cereus* showed maximum lactonase activity. Further, the culture filtrates of two endophytic bacteria *Bacillus firmus* PT18 and *Enterobacter asburiae* PT39 exhibited strong quorum sensing inhibition by reducing violacein production by 80%. These culture filtrates also showed strong inhibition of biofilm in *Pseudomonas aeruginosa* (Rajesh and Rai 2014). Similarly, endophytic *Bacillus megaterium* and *Brevibacillus borstelensis* and two *Bacillus* sp. isolated from *Cannabis sativa* also showed quorum sensing inhibition by reducing violacein production (Kusari et al. 2014). Bacterial endophytes *Microbacterium testaceum* BAC1065, BAC1100, and BAC2153, *Bacillus thuringiensis* BAC3151, and *Rhodococcus erythropolis* BAC2162 also exhibited quorum quenching activity against *Pseudomonas syringae* and *Hafnia alvei* (Lopes et al. 2015). Recently, culture filtrate of endophytic bacterium *Bacillus cereus* displayed strong quorum sensing inhibitory activity against *Pseudomonas aeruginosa* and *Pectobacterium carotovorum* (Rajesh and Rai 2016).

Fungal endophytes such as *Fusarium graminearum* and *Lasiodiplodia* sp. showed decreased production of violacein which suggested antiquorum activity (Rajesh and Rai 2013). Similarly, the biomass and cell-free extract of marine endophytes *Sarocladium* sp. (LAEE06), *Fusarium* sp. (LAEE13), *Epicoccum* sp. (LAEE14), and *Khuskia* sp. (LAEE 21) strongly suppressed violacein production by 70% (Martin-Rodriguez et al. 2014). Further, polyhydroxyanthraquinones produced by *Penicillium restrictum* act as a quorum sensing inhibitor against the spectrum of methicillin-resistant *Staphylococcus aureus* with an IC_{50} value of 8–120 μM

(Table 4.2) (Figueroa et al. 2014). Thus, quorum sensing inhibitors from endophytes can be a useful agent in both biocontrol and clinical arena.

4.3.7 Urease Inhibitors

Urease is an enzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide. The enzyme accelerates the reaction by 100 trillion-fold as compared to nonenzymatic reaction. Ureases are important virulence factor in the gastrointestinal and urinary tract infections caused by *Helicobacter pylori* or various *Proteus* species. Infections caused by ureolytic bacteria lead to serious health problems like pyelonephritis, hepatic coma, peptic ulcer, and kidney stones (Upadhyay 2012; Modolo et al. 2015; Khan et al. 2015). Urease inhibitors are molecules that suppress the hydrolytic action of urease. Urease inhibitors are found to dissolve kidney stone and also prevent the formation of new crystals in urine. They are also considered as potential targets of antiulcer drug. Until now, only one compound, acetohydroxamic acid has been clinically approved for treatment of urinary tract infection in which the patient also suffers from several side effects. Thus, there is a requirement for development of novel, selective, and efficient urease inhibitors which could assure the requirements of low toxicity and cost-effectiveness (Kosikowska and Berlicki 2011; Macegoniuk 2013).

The study carried out by Haroon et al. (2014) demonstrated that the ethyl acetate extracts of marine-derived endophytic fungus *Aspergillus terreus* exhibited potential urease inhibitory activity with an IC_{50} value of 116.8 μM. Further, a new radicinol derivative, bipolarisenol, isolated from the ethyl acetate extract of endophytic fungus *Bipolaris sorokiniana* LK12 also showed promising urease inhibition in a dose-dependent way with an IC_{50} value of 81.62 μg/ml. The compound also possessed acetyl cholinesterase and lipid peroxidation inhibitory properties (Table 4.2, Fig. 4.1) (Khan et al. 2015). Similarly, sorokiniol isolated from the same fungus also displayed 50% urease inhibition (Ali et al. 2016). Recently, fungal endophytes isolated from *Boswellia sacra* also exhibited urease inhibition. Isolate *Fusarium oxysporum* FEF1, *Penicillium spinulosum* FEF2, *Aspergillus caespitosus* FEF3, *Alternaria alternata* FEF5, and *Penicillium citrinum* FEF6 showed 45–85% inhibitory activity on urease. Further, the organic extract of *Penicillium citrinum* FEF6 was fractionized into five different compounds which showed moderate urease inhibitory activity (20–40%) which clearly depicted that the isolate possessed synergistic inhibitory activity (Ali et al. 2017). The present reports are scanty and preliminary, and it requires extensive research for the development of new urease inhibitors from endophytic microorganisms.

4.3.8 Protease Inhibitors

Protease inhibitors (PIs) are lead molecules that block the activity of protein-digesting enzymes, “proteases,” involved in viral replication and pathogenesis. PIs

check viral replication by selectively binding to viral proteases, thereby blocking the hydrolytic cleavage of precursor proteins, essential for production of pathogenesis (Ghosh et al. 2016). Thus, PIs play a significant role in the treatment of viral diseases including human immunodeficiency virus (HIV), herpesvirus, and hepatitis C virus (HCV). Although several PIs like saquinavir, nelfinavir, ritonavir, and atazanavir are already available for clinical use, emergence of toxicity, new recombinant viral strains, and drug resistance have daunting effect on current antiviral therapy. Thus, development of new antiviral drugs is the need of the hour to deal with the present scenario (Singh et al. 2004; Roy 2017).

Natural products have always been the mainstay of structurally diverse bioactive secondary metabolites. Several potential antiviral compounds have been reported from endophytic fungi. Two *p*-tridepside derivatives, cytonic acids A and B, isolated from endophytic *Cytonaema* sp. exhibited human cytomegalovirus protease inhibitory activity with an IC_{50} values of 43 and 11 μmol, respectively (Table 4.2, Fig. 4.1; Guo et al. 2000). Singh et al. (2004) reported the production of hinnuliquinone, a potential inhibitor of HIV-1 protease from endophytic fungi inhabiting leaves of oak tree. The compound showed strong inhibition of protease isolated from drug-resistant wild-type mutant strain of HIV (A-44) with an IC_{50} values of 2.5 and 1.8 μM, respectively. (+)-Sclerotiorin isolated from the hexane extract of endophytic *Penicillium sclerotiorum* PSU-A13 also displayed inhibitory effect on the HIV-1 protease with an IC_{50} of 62.7 μg/mL (Arunpanichlert et al. 2010). Further, pestalotheols A–D isolated from endophytic *Pestalotiopsis theae* were also tested for their inhibitory activity of HIV-1 replication. It was observed that among the four metabolites, pestalotheol C exhibited inhibitory effect on HIV-1 replication in C8166 cells with an IC_{50} value of 16.1 μM (Li et al. 2008). Similarly, altertoxins (V, I, II, and III) isolated from endophytic *Alternaria tenuissima* QUE1Se completely inhibited replication of HIV-1 virus (Bashyal et al. 2014). Govindappa et al. (2015) reported that the organic extract of *Alternaria* sp., *Fusarium* sp., and *Trichoderma harzianum* inhibited the activity of HIV reverse transcriptase, integrase, and protease enzymes, respectively.

Anthraquinones isolated from endophytic marine fungus *Aspergillus versicolor* showed inhibition of HCV protease. The ethyl acetate extract along with isorhodoptilometrin-1-methyl ether, emodin, 8-methyl-emodin, siderin, arugosin C, and variculanol inhibited hepatitis C virus NS3 protease (Hawas et al. 2012). Similarly, alternariol derivatives obtained from the extract of *Alternaria alternata* displayed high-level inhibition of HCV. The ethyl acetate extract, alternariol, and maculosin depicted strong inhibition of HCV NS3/4A protease with IC_{50} values of 14, 32.2, and 12 μg/ml, respectively (Hawas et al. 2015). Recently, endophytic *Penicillium chrysogenum* isolated from red alga *Liagora viscida* also showed potential inhibitory activity toward HCV NS3/4A protease with an IC_{50} value of 20 μg/mL (Hawas et al. 2013). Further, the culture metabolites obtained from marine endophytic *Fusarium* sp. were capable of inhibiting hepatitis C virus NS3/4A protease. Among the tested compounds, ω-hydroxyemodin and griseoxanthone C showed maximum inhibition with IC_{50} values of 10.7 and 19.8 μM, respectively (Hawas et al. 2016). Thus, endophytes appear to be a promising source of novel

antiviral metabolites. However, the number of antiviral compounds reported to date is very handful, and there is a need to search for newer biotypes from different ecological niches, which could produce novel lead molecules with potential antiviral activity.

4.3.9 DNA Polymerase Inhibitors

DNA polymerases are enzymes that synthesize DNA. Human genome encodes for about 16 types of polymerases that are involved in highly regulated functions like DNA synthesis, repair, and recombination. Eukaryotic cell comprises of 3 replicative polymerases (α, δ, and ε), a mitochondrial polymerase (γ), and 11 non-replicative polymerases (β, ξ, η, θ, ι, κ, λ, μ, ν, terminal deoxynucleotidyl transferase, and REV1). Based on their sequence similarity, eukaryotic polymerases are classified into four families A, B, X, and Y. Family A contains mitochondrial polymerase γ and non-replicative polymerases θ and ν, whereas family B includes three replicative polymerases (α, δ, and ε) and non-replicative polymerase ξ. Family X comprises of non-replicative polymerases β, λ, and μ and terminal deoxynucleotidyl transferase, whereas family Y contains polymerases η, ι, κ, and REV1 (Kamisuki et al. 2007; Kimura et al. 2008; Nishida et al. 2008).

Numerous pathological conditions like cancer, autoimmune disorders, and bacterial or viral infections are often caused due to uncontrolled DNA replication. Inhibition of this vital biological process provides an obvious management strategy against these diseases (Berdis 2008). Solanapyrone A, isolated from fungus SUT 01B1-2, selectively inhibits DNA polymerases β and λ with an IC_{50} values of 30 and 37 μM, respectively (Table 4.2, Fig. 4.1) (Mizushina et al. 2001). Similarly, kasanosins A and B isolated from the culture filtrates of marine-derived *Talaromyces* sp. also inhibited β and λ DNA polymerases in a dose-dependent way. Kasanosins A showed comparatively strong inhibition of rat polymerase β and human polymerase λ with IC_{50} values of 27.3 and 35 μM, respectively (Kimura et al. 2008). Hymenoic acid produced by the coral-derived fungus *Hymenochaetaceae* sp. exclusively inhibited λ DNA polymerase with an IC_{50} value of 91.7 μM in a noncompetitive manner (Nishida et al. 2008). Further, trichoderonic acids A and B and (+)-heptelidic acid isolated from *Trichoderma virens* IG34HB competitively suppressed the activity of mammalian non-replicative DNA polymerases β, λ, and terminal deoxynucleotidyl transferase (Yamaguchi et al. 2010).

1-deoxyrubralactone isolated by the fungal strain HJ33 derived from sea algae selectively inhibited X and Y families of eukaryotic DNA polymerase with an IC_{50} values of 11.9–59.8 μM, respectively (Naganum et al. 2008). Further, *Penicillium daleae* isolated from sea moss produced Penicilliols A and B which exclusively inhibited Y family of mammalian DNA polymerase with IC_{50} values of 19.8–32.5 μM, respectively (kimura et al. 2009). Another *Penicillium* sp. from seaweed produced pinophilins A and B which potentially inhibited A, B, and Y families of DNA polymerase. Pinophilins A exhibited strongest inhibition in a noncompetitive manner with IC_{50} values of 48.6–55.6 μM. Thus, selective polymerase inhibitors are

considered as a feasible candidate in chemotherapy because many of them can inhibit human cancer cell proliferation and were also found to be cytotoxic (Myobatake et al. 2012).

4.3.10 Trypanothione Reductase Inhibitor

Protozoan parasites like *Trypanosoma* and *Leishmania* found at the tropical and subtropical regions of the world affect millions of people resulting in massive medical, economic, and social loss in the affected area (Beig et al. 2015; Campos et al. 2015; Fatima et al. 2016a, b). The World Health Organization (WHO) has listed all the diseases caused by these parasites among neglected tropical disease. The current arsenal of drugs available in the market for the treatment of different forms of leishmaniasis and trypanosomiasis were introduced several decades ago and has significant drawbacks like efficacy, toxicity, drug resistance, and cost-effectiveness. Hence, there is an utmost requirement of finding out new drugs with better efficacy and lower toxicity (Campos et al. 2008; Cota et al. 2008). Trypanothione reductase is an enzyme found in several trypanosomatids including *Leishmania* and *Trypanosoma* spp. Trypanothione reductase is involved in the protection of *Trypanosoma* and *Leishmania* sp. against oxidative stress and is considered as a potential drug target for treatment against trypanosomatids (Garrard et al. 2000; Beig et al. 2015).

Alentusin, a biphenyl derivative isolated from the organic extract of endophytic fungus *Alternaria* sp. UFMGCB55 significantly inhibited 99% of trypanothione reductase with an IC_{50} value of 4.3 μM (Table 4.2, Fig. 4.1) (Cota et al. 2008). Organic extract of endophytic *Cochliobolus* sp. exhibited 90% inhibition of *Leishmania amazonensis* and 100% reduction of Ellman's reagent in trypanothione reductase assay under in vitro conditions. Further, the fractionation of the extract eluted two compounds, cochlioquinone A and isocochlioquinone A, both of which were active against *L. amazonensis* with an EC_{50} value of 1.7 and 4.1 μM, respectively (Campos et al. 2008). Rosa et al. (2010) screened 121 isolates obtained from various Brazilian forests for leishmanicidal and trypanocidal activity. The ethyl acetate extract of 11 isolates inhibited *L. amazonensis* with an IC_{50} values ranging from 4.6 to 24.4 μg/ml. Endophytic isolate UFMCB 529 and 910 exhibited 90% inhibition in the growth of *L. amazonensis*. Further, 24 isolates displayed inhibition of trypanothione reductase, while only 3 of them showed inhibitory effect (>60%) on the growth of *Trypanosoma cruzi* with an IC_{50} values of 1–10 μg/ml. Extract of endophytic isolates UFMCB 508, 509, 513, 529, 563, 579, and 648 inhibited trypanothione reductase and was also active against the amastigote forms of *L. amazonensis*. Isolate UFMCB 508 displayed comparable activity with benznidazole, an antiparasitic medication used in Chagas disease (Rosa et al. 2010). Furthermore, over 560 endophytic isolates recovered from Antarctic angiosperms *Deschampsia antarctica* were also screened for their leishmanicidal activity. Extract of 12 isolates checked the proliferation of *L. amazonensis* with IC_{50} values ranging from 0.2 to 125 μg/ml. Further, *Alternaria*, *Cadophora*, *Herpotrichia*, and *Phaeosphaeria* spp.

showed >90% killing of *L. amazonensis*. It will be interesting to examine whether extracts derived from endophytic isolates possess leishmanicidal activity via trypanothione reductase inhibition or not (Santiago et al. 2012). Recently, Fatima et al. (2016a, b) used in silico approach to study antileishmanial activity of epicoccamide derivatives A–D (endophytic origin). The study revealed that epicoccamide derivatives were stabilized at the active site of the enzyme via hydrogen bond and hydrophobic interactions. Epicoccamide derivatives depicted high binding energies with trypanothione reductase with binding energies of -13.31, -13.44, -13.31, and -13.32 Kcal/mol, respectively. Thus, trypanothione reductase inhibitors from endophytic isolates could serve as novel lead molecules in the management of neglected tropical diseases like trypanosomiasis and leishmaniasis.

4.3.11 DNA Topoisomerase Inhibitors

DNA topoisomerases are crucial enzymes that play a significant role in DNA replication and cell division. They are involved in uncoiling and recoiling of DNA. Based on their catalytic mode of action, they are classified into two different types: topoisomerase I and topoisomerase II. Topoisomerase I relaxes DNA supercoiling during replication and transcription by transiently creating a single-strand nick in the DNA, whereas topoisomerase II acts by making a transient double-strand breaks in DNA. Topoisomerase is recognized as target for anticancer drugs (Pommier 2009; Jarolim et al. 2017). The inhibitors block the activity of topoisomerase to bind the DNA back together after it has been cut, making the enzyme nonfunctional. Topoisomerase inhibitors have the ability to kill cells undergoing DNA replication, stop translation of DNA for protein production, and prevent DNA damage and repair. Since, cancer cell proliferates more rapidly than the normal cells, and the cancer cells will be disproportionately killed by the topoisomerase inhibitors. Topoisomerase inhibitor I includes camptothecin, whereas topoisomerase inhibitor II comprises of doxorubicin and etoposides which have displayed remarkable therapeutic potential against certain cancers including breast, bladder, colon, uterine, cervical, and ovarian cancer (Kusari et al. 2009a; Baikar and Malpathak 2010). Camptothecin and its derivatives are the third largest anticancer drugs. Both camptothecin and podophyllotoxin (precursor of etoposides) are plant products originally isolated from the *Camptotheca acuminata* and *Podophyllum* sp., respectively. The huge market demand caused large-scale destruction of source plants from their natural environment resulting into endangered species status of the plants. Further, toxicity, short half-life, and cellular uptake were some important shortcomings related to them. Hence, alternative sources need to be exploited to meet the global market demand with effective therapeutic potential (Puri et al. 2006; Pu et al. 2013).

Endophytes have been reported as prolific producers of anticancer agents. The discovery of billion-dollar anticancer drug paclitaxel from *Taxomyces andreanae*, an endophyte of *Taxus brevifolia*, was a breakthrough discovery in endophytic research (Stierle et al. 1993). Since then, many anticancer agents have been isolated from various endophytic fungi. Puri et al. (2005) first reported the production of

camptothecin from endophytic *Entrophospora infrequens* obtained from *Nothapodytes foetida*. Further, Kusari et al. (2009a) isolated a camptothecin and its derivatives producing endophytic fungus *Fusarium solani* from *Camptotheca acuminata*. Furthermore, camptothecin-producing endophytic *Aspergillus* sp. LY341, *Aspergillus* sp. LY355, and *Trichoderma atroviride* LY357 were also isolated from *Camptotheca acuminata* collected from campus of the Chengdu Institute of Biology of the Chinese Academy of Sciences, Chengdu, China (Pu et al. 2013). Similarly, Shweta et al. (2010) also documented the production of camptothecin, hydroxycamptothecin, and 9-methoxycamptothecin from endophytic *Fusarium solani* isolated from *Apodytes dimidiata*. Apart from endophytic fungi, camptothecin and its derivative 9-methoxy camptothecin production were also observed in endophytic bacteria isolated from *Miquelia dentata* (Shweta et al. 2013).

Podophyllotoxin is the precursor for chemical synthesis of anticancer drugs like etoposide and teniposide (topoisomerase II inhibitors) that are used in breast, lung, and testicular cancer therapy. Yang et al. (2003) first reported the production of podophyllotoxin from six endophytic fungi isolated from *Sinopodophyllum hexandrum*, *Diphylleia sinensis*, and *Dysosma veitchii*. Later, Eyberger et al. (2006) isolated two strains of endophytic *Phialocephala fortinii* PPE5 and *Phialocephala fortinii* PPE7 that possessed the ability to produce podophyllotoxin. Similarly, podophyllotoxin and its glycoside production were also detected in the Sabouraud broth culture of endophytic *Trametes hirsute* isolated from *Sinopodophyllum hexandrum* (Puri et al. 2006). Furthermore, endophytic *Fusarium oxysporum* and *Aspergillus fumigates* isolated from *Juniperus communis* also exhibited production of podophyllotoxin (Kour et al. 2008; Kusari et al. 2009b). The above reports suggest that endophytes could be a promising natural resource for obtaining camptothecin, podophyllotoxin, and their derivatives.

Apart from producing anticancer molecules like camptothecin and podophyllotoxin, endophytes are also documented to exhibit inhibition of topoisomerase enzymes. Guo et al. (2007) first reported the inhibition of topoisomerase I by secalonic D produced from endophytic *Paecilomyces* species. Similarly, Xiaoling et al. (2010) screened ethyl acetate extract of 56 endophytic fungi isolated from mangrove plants in Qi'ao island of Zhuhai, China, among which extract of 19 fungal isolates showed topoisomerase I inhibitory activity. Further, Shino et al. (2007) reported production of fusaristatins B, a new cyclic lipopeptides from an endophytic *Fusarium* sp. that appreciably inhibited topoisomerase I and II with an IC_{50} values of 73 μM and 98 μM, respectively (Table 4.2, Fig. 4.1). Similarly, aspergiloid I, produced by endophytic *Aspergillus* sp. YXf3, possessed the ability to inhibit topoisomerase II (Guo et al. 2014). Thus, from the above reports, it clearly becomes evident that endophytes are promising alternative source of topoisomerase inhibitors which can be developed as a potential anticancer agents.

4.3.12 Aromatase Inhibitor

Breast cancer is one of the foremost causes of mortality in women around the world. Every one in eight women in America is expected to be diagnosed with breast cancer in her lifetime. Tumor cell proliferation is stimulated by the circulating estrogen; that is why over 75% of the patients diagnosed with breast cancer have estrogen-dependent breast cancer. In breast cancer tissues, an increased level of enzyme aromatase was found around the tumor site (Chomcheon et al. 2009; Fatima et al. 2014). Aromatase is an enzyme that carries out the catalytic conversion of androgens into estrogens. Thus by ceasing the activity of the aromatase enzyme, 90% of the estrogen production can be reduced which will significantly reduce the chances of breast cancer. Presently, aromatase inhibitors like letrozole and exemestane are used as hormonal therapy in patients with estrogen-dependent postmenopausal breast cancer (Altundag and Ibrahim 2006; Sureram et al. 2012; Chottanapund et al. 2017). Thus, aromatase inhibitors appear to be a plausible target for treatment of estrogen-dependent breast cancer, and new avenues need to be explored for finding out novel aromatase inhibitors.

Corynesidone A isolated from the broth extract of endophytic fungus *Corynespora cassiicola* exhibited aromatase inhibitory activity with an IC_{50} value of 5.3 μM (Table 4.2, Fig. 4.1). The compound also showed strong antioxidant activity (Chomcheon et al. 2009). Similarly, isocoumarins and phthalide extracted from the culture filtrate of the endophytic fungus *Colletotrichum* sp. CRI535-02 were also capable of inhibiting aromatase enzyme with an IC_{50} ranging from 15.3 to 16.9 μM (Tianpanich et al. 2011). Further, azaphilone derivative derived from the endophytic fungus *Dothideomycetes* sp. CRI7 also showed aromatase inhibitory activity with an IC_{50} value of 12.3 μM (Hewage et al. 2014). Recently, two endophytic isolates *Epicoccum nigrum* and *Penicillium* sp. isolated from west Himalayan yew *Taxus fuana* exhibited 73–76% aromatase inhibition with IC_{50} values of 12.2 and 10.5 μg/ml, respectively (Fatima et al. 2016a, b).

Depsidones produced by a marine-derived fungus *Aspergillus unguis* CRI282-03 were capable of inhibiting aromatase enzyme with IC_{50} values of 1.2–11.2 μM, respectively (Sureram et al. 2012). Further, two despidones, unguinol and aspergillusidone A, were also tested for their antiaromatase activity against human primary breast adipose fibroblasts and hormonal-responsive T47D breast tumor cells. It was found that despidones inhibited the growth of T47D breast tumor cells via inhibition of aromatase activity with an IC_{50} of 9.7 and 7.3 μM, respectively (Chottanapund et al. 2017). Thus, endophytic fungi appear to be a unique natural bioresource of aromatase inhibitors with huge possibilities in breast cancer therapy.

4.3.13 α-Amylase and α-Glucosidase Inhibitors

Diabetes mellitus (DM) is a serious global health problem characterized by chronic hyperglycemia and disturbed carbohydrate, fat, and protein metabolism (Indrianingsiha and Tachibana 2017; Ruzieva et al. 2017). DM is linked with other

complications like cardiovascular disorders, retinopathy, nephropathy, and neuropathy (El-Hady et al. 2014). The number of people suffering from DM is alarming, and it is believed that about 522 million peoples will be affected by the year 2030. India is expected to have maximum number of diabetes patients in the coming years (Akshatha et al. 2014; Pavithra et al. 2014; Singh and Kaur 2015). Type 2 diabetes is the most prevalent type of diabetes, with >90% of people suffering from it. Postprandial hyperglycemia is a major risk factor involved in type 2 diabetes. The elevated level of postprandial hyperglycemia is attributed to the action of two carbohydrate-hydrolyzing enzymes, viz., α-amylase and α-glucosidase. The enzymes are involved in breakdown complex sugar moieties into more simpler and absorbable form, leading to increased blood sugar level. One of the management strategies of DM involves inhibition of these enzymes. Inhibition of these enzymes slows down the rate of carbohydrate digestion and glucose absorption, ultimately lowering hyperglycemia. Thus, inhibition of α-glucosidase and α-amylase appears to be an effective target for diabetes management (Pujiyanto et al. 2012; Xia et al. 2015; Ruzieva et al. 2017). The oral antidiabetic drugs like acarbose and miglitol are inhibitors of α-glucosidase. However, these agents are synthetic in origin and suffer from various adverse effects like flatulence, abdominal pain, renal tumors, hepatic injury, etc. (Pavithra et al. 2014). These synthetic drugs need to be replaced with drugs of natural origin that are believed to have lesser or no side effects.

The recent studies suggested that endophytic microorganisms offer themselves as magnificent producers of α-glucosidase and α-amylase inhibitors. Endophytic actinomycetes isolated from various Indonesian diabetic plant species exhibited α-glucosidase inhibitory activity. Among the screened actinomycetes, *Streptomyces olivochromogenes* BWA65 obtained from *Tinospora crispa* displayed maximum α-glucosidase inhibition (Pujiyanto et al. 2012). Similarly, Akshatha et al. (2014) reported that extract of *Streptomyces longisporoflavus* competently inhibited α-amylase with an IC_{50} value of 162 μg/mL.

Several marine-derived fungi were also reported to possess antidiabetic property. Eremophilane sesquiterpenes isolated from endophytic *Xylaria* sp. inhibited α-glucosidase with an IC_{50} value of 6.54 μM (Song et al. 2012). The mycelial and culture filtrate extract of a coral-derived fungus *Emericella unguis* 8429 also displayed 51 and 64% inhibition of α-glucosidase enzyme (El-Hady et al. 2014). Similarly, isopimarane diterpene and 11-deoxydiaporthein A produced from *Epicoccum* sp. HS-1 also demonstrated α-glucosidase inhibitory activity with IC_{50} values of 4.6 and 11.9 μM, respectively (Xia et al. 2015).

Endophytic fungi from terrestrial plants are considered as lucrative source of antidiabetic agents. Ramdanis et al. (2012) screened endophytic fungi isolated from the seeds of *Swietenia macrophylla* for α-glucosidase inhibitors. During the study, five isolates showed α-glucosidase inhibitory activity. The IC_{50} value of most potent isolate CMM4B (73.64 μg/ml) was found to be better than that of acarbose (117.06 μg/ml) under in vitro conditions. Similarly, endophytic *Colletotrichum* sp. isolated from *Taxus sumatrana* showed 71% inhibition of α-glucosidase (Artanti et al. 2012). Thielavins A, J, and K isolated from endophytic fungal isolate MEXU 27095 inhibited α-glucosidase in a dose-dependent way with IC_{50} values of 23.8, 15.8, and

22.1μM, respectively (Rivera-Chavez et al. 2013). Recently, Indrianingsiha and Tachibana (2017) reported production of a potential α-glucosidase inhibitor from Xylariaceae sp.QGS01. Similarly, Ali et al. (2017) also determined α-glucosidase inhibitory activity of an endophytic *Penicillium citrinum* isolated from *Boswellia sacra*. Furthermore, Pavithra et al. (2014) screened extract of 22 endophytic fungal isolates obtained from *Momordica charantia* and *Trigonella foenum-graceum* for α-amylase, α-glucosidase, and aldose reductase inhibitory activity. Isolate *Stemphylium globuliferum* PTFL005 and PTFL011 displayed α-glucosidase inhibitory activity with IC_{50} values of 17.37 and 10.71 μg/mL, whereas isolate *Stemphylium globuliferum* PTFL005 and PTFL006 showed promising α-amylase inhibitory activity with an IC_{50} values of 15.48 and 13.48 μg/ml, respectively. Further, *Trichoderma atroviride* PMCF003 displayed moderate aldose reductase inhibitory property. Recently, fungal endophytes isolated from the medicinal diabetic plants of Uzbekistan were also screened for their α-amylase inhibitory activity. The screened isolates showed 60–82% inhibition of α-amylase (Ruzieva et al. 2017). Peptides produced by endophytic *Aspergillus awamori* exhibited both α-amylase and α-glucosidase inhibitory activity with low IC_{50} values of 3.75 and 5.62 μg/mL, respectively. The inhibitor was stable over a range of high and low pH and temperature and was non-mutagenic in nature (Singh and Kaur 2015). The above reports suggest that endophytes can be harnessed as new α-amylase and α-glucosidase inhibitors for the better management of diabetes.

4.3.14 Pancreatic Lipase Inhibitors

Obesity is a burgeoning health concern which occurs due to an imbalance between calorie uptake and utilization. Today, obesity is becoming the major cause of preventable deaths, both in developed and developing nations. It has been reported that every third individual around the globe is obese. Further, it has also been projected that if the current scenario continues, by the end of year 2020, every two individuals out of three will be overweight or obese (Fitri et al. 2017; Katoch et al. 2017). The management of obesity can be done by two different anti-obesity therapies including exercise and/or drug therapy. Drug therapy is more convincing as there is relapse of weight gain after physical activity has been stopped. Drug therapy includes targeting drugs to central or peripheral nervous system eventually leading to loss of hunger and lipase inhibition (Lunagariya et al. 2014). Pancreatic lipase (PL) is the key enzyme involved in lipid metabolism. PL hydrolyzes about 50–70% of the triglycerides resulting in the formation of monomers of fatty acids that are absorbed and accumulated in the body resulting to obesity (Gupta et al. 2014; Sharma et al. 2017). Hence, PL appears to be suitable target for obesity management. Orlistat isolated from actinobacterium *Streptomyces toxytricini* is one of the best-selling (PL inhibitor) anti-obesity drug. However, it also suffers from several side effects like oily stools, flatulence, fecal urgency, and abdominal cramps. Thus, alternative avenues need to be explored for isolation of novel PL inhibitors with low or no side effects (Gupta et al. 2015).

Natural products either from plants or microorganisms offer themselves as potential source of PL inhibitor. Many natural products have been reported to exhibit inhibition of PL. However, very preliminary reports are available on PL inhibitors from endophytic microorganisms. Gupta et al. (2014) first reported PL inhibitors from endophytic fungi. A screening program was designed to screen endophytic fungi from *Aegle marmelos* collected from biodiversity hot spots of India. Among the screened fungi, endophytic *Fusarium incarnatum* (#6AMLWLS), *Botryosphaeria stevensii* (#59 AMSTWLS), and *Fusarium semitectum* (#1058 AMSTITYEL) showed maximum inhibition of PL. Further, the IC_{50} value of aqueous extract of *F. incarnatum* was 2.12 μg/ml which was better than commercially available drug orlistat (2.79 μg/ml) under in vitro conditions. The lead molecule was further purified and characterized using analytical and biochemical tools and was identified as a novel tetrapeptide "Fustat" (patent filing under process) (Table 4.2). Similarly, culture filtrates obtained from endophytic fungi isolated from medicinal plants like *Cinnamomum camphora*, *C. zeylanicum*, *Camellia sinensis*, *Piper nigrum*, and *Taxus baccata* were also screened for PL inhibitory activity. The chromogenic plate assays indicated that endophytic fungal isolate #57 TBBALM (*Penicillium* sp.), #33 TBBALM (*Mycelia sterilia*), and #1 CSSTOT (*Schizophyllum* sp.) exhibited maximum inhibition of PL. Further, the IC_{50} value of organic extract of #57 TBBALM (3.69 μg/ml) was also found to be comparable with orlistat (2.73 μg/ml) (Gupta et al. 2015). Recently, Katoch et al. (2017) reported inhibition of PL by the crude extracts of endophytic fungi obtained from *Viola odorata*. Among the tested fungi, ten isolates showed potential inhibition of PL with IC_{50} value >1 μg/ml. *Aspergillus* sp. VOLF4 exhibited promising PL inhibition with an IC_{50} value of 3.8 μg/ml. Apart from endophytic fungi, PL inhibitory activity of endophytic actinobacteria has also been recently reported. Endophytic *Streptomyces* isolates AEBg4, AEBg10, AEBg12, AELk3, and AEKp9 isolated from various Indonesian medical plants showed significant inhibition (92–96%) of PL (Fitri et al. 2017). The above reports suggest that endophytic isolates are promising source of PL inhibitors. However a more detailed, rationalized, and target-based studies are required before moving to preclinical trials.

4.4 Conclusion

Endophytes are considered as a rich source of structurally diverse bioactive metabolites having potential application in agriculture, pharmaceutical, and food industry. However, looking at the humongous biodiversity of endophytic microorganisms, it seems that they still remain an underexplored resource of enzyme inhibitors. The published reports are scanty, and issues like low productivity, toxicity, cellular uptake, and short half-life need to be resolved first. The advances made in the field of modern biotechnology such as genetic engineering and microbial fermentation technology should be taken into consideration for better understanding and successive manipulation of endophytic microorganism and to make it more beneficial for the mankind. The first step toward this approach is exploration of a potential

candidate from the natural environment. Further, through fusion, mutation, recombination, and genetic manipulations, the viable candidate should be selected for large-scale fermentation. The strategy promises to improve the production of therapeutically important enzyme inhibitors at cheaper and more affordable cost. Apart from this, there is a need among different scientific disciplines (microbiologist, chemist, toxicologist, and pharmacologist) to work in a coordinated manner for the discovery of the target lead molecule. If we will be able to achieve the above mentioned targets, enzyme inhibitors from endophytic microorganisms will emerge as a future medicine which can be used to cure all major health problems.

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5 Significance and Approaches of Microbial Bioremediation in Sustainable Development

Arvind Kumar, Sruchi Devi, and Digvijay Singh

Abstract

Microorganisms like bacteria, yeast, or fungi have the capabilities to degrade or transform hazardous chemicals like benzene, toluene, polychlorinated biphenyls (PCB), dioxins, etc. into nontoxic or less toxic substances, known as microbial bioremediation. It has been used for treating contaminated water and soil. It involves the promotion of growth of specific microflora which is local to that contaminated sites. Various approaches can be used for promoting the growth of microflora like incorporation of nutrients and addition of electron acceptors molecules or controlling temperature and moisture. Contaminants play the role of nutrients for microorganisms in bioremediation. Generally it involves biodegradation and biotransformation which convert the hazardous substances to non-hazardous or less-hazardous form with the production of carbon dioxide or methane, water, and biomass. Microorganisms which are considered for bioremediation are *Actinobacteria*, *Alcaligenes*, *Flavobacterium*, *Mycobacterium*, *Mycococcus*, *Nitrosomonas*, *Penicillium*, *Phanerochaete*, *Pseudomonas*, *Serratio*, *Trametes*, and *Xanthobacter.* Many petroleum hydrocarbons like benzene, toluene, and O-xylene (BTX) have been biodegraded by *Pseudomonas putida* MHF7109. *Pseudomonas nitroreducens* PS-2 has been found useful for bioremediation of pesticide chlorpyrifos. Cow dung microflora has been found for bioremediation for benzene. Treatment technologies are being used; still organic pollutants are existing in the soil and water beyond their normal limit; hence bioremediation along with recombinant DNA technology can be a significant tool to remove toxic substances.

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5.1 Introduction

The use of biological microorganisms to metabolize contaminants by natural way in the environment is called bioremediation. This treatment facility can be accomplished in both on-site (in situ) and off-site (ex situ). Bioremediation is better than other remediation methods as it is cheap, more efficient, and less likely to produce toxic intermediates to contaminant removal (Paul et al. 2005; US EPA 2001). Bioremediation has been successfully employed in treating soil, sludge, and groundwater for chlorinated volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), pesticides, and herbicides (US EPA 2001). Microorganisms are indicators of a healthy environment diverse, versatile, and exhibit an ability to adapt any environment. Disturbances of ecosystem can cause lower diversity of microbial populations (Shade et al. 2012; Wertz et al. 2007). There are some extremely low diversity systems found in highly impacted environments such as acid mine drainage (AMD) sites (Andersson and Banfield 2008; Baker and Banfield 2003; Baker et al. 2009). Observation of changes in microbial community profiles helps to determine whether bioremediation is naturally occurring in an environment; this process is called natural attenuation (Desai et al. 2009; Dojka et al. 1998). For example, natural attenuation was observed in the Gulf of Mexico after the 2010 Deepwater Horizon oil spill when environmental microbes capable of degrading hydrocarbons were found to increase after the accidental introduction of approximately 4.9 million barrels of crude oil into the marine ecosystem (Kostka et al. 2011; Lu et al. 2011; Mason et al. 2012). Microbial community monitoring can be used to determine which microbial species predominate in contaminated areas if bioremediation efforts are successful.

For bioremediation microorganisms are excellent candidates. Some species naturally possess the ability to metabolize various xenobiotics (Eyers et al. 2004). Xenobiotics are foreign compounds to living organisms that tend to accumulate in natural water or soil environments. They are not easily degradable by most biological enzymes (Eyers et al. 2004). These compounds can be toxic, mutagenic, or even carcinogenic for many living organisms. These toxic compounds and contaminant are classified as metals, nonmetals, metalloids, inorganic salts, and organic molecules. Organic contaminants can be further divided into aromatic, aliphatic, alicyclic, and polycyclic aromatic hydrocarbon, which also contain halogenated and non-halogenated molecules, explosives, and pesticides. Some inorganic compounds includes heavy metals such as Pb, Al, Ag, As, Cd, Be, Cr, Hg, Cu, Sb, Fe, Ni, Zn, and Se along with few radioactive substances with their derivatives.

In these days many physical, chemical, and biological processes are used to treat and manage contaminants. RCRA of 1976 with amendments in 1986 (The Resource Conservation and Recovery Act) explains the issues of generation of contaminants, their transportation, treatment before to release, storage space, and dumping of hazardous waste. Moreover, hard regulatory standards forced in different countries for decontamination of contaminated waste dump areas have directed the researchers to search different methodology in bioremediation. The RCRA advises for development of eco-friendly methods such as bioremediation or natural attenuation. Bioremediation deals with the deprivation of pollutants by transforming them into nontoxic or less toxic substance, particularly water and carbon dioxide. This is carried out moreover on bioremediation site by using native microorganisms or preface of strain of bacteria or fungal and in ex situ bioremediation to accomplish complete detoxification of contaminants.

Recent study is showing that bioremediation is more appropriate method of remediating soils, very effective and also cheaper as compared with physicochemical methods. Bioremediation processes have many advantages compared to other soil remediation processes (solvent extraction, adding oxidizing agents, etc.) making it an effective method of treating polluted environments (Gogoi et al. 2003).

In situ bioremediation is based on the activity of microorganisms to use petroleum hydrocarbons as a source of carbon and energy. It is considered to be the most significant method because it is not having any irreversible effects on soil health and also cost effective. Microorganisms such as bacteria, cyanobacteria, yeasts, and fungi break down these dangerous chemicals into less toxic or nontoxic compounds. To survive, microorganisms need nutrients (such as nitrogen, phosphorus, potassium, trace elements), carbon, and energy source. Microorganisms which are found naturally in soil transform various organic compounds into food and energy for their own ecosystem. For example, many bacterial species from the soil can use petroleum hydrocarbons as a source of energy and food. This natural method transforms petroleum hydrocarbons in less toxic substances such as carbon dioxide, water, and fatty acids. Degradation of organic waste compounds has a major methodology for treatment of polluted soils (Atlas 1992; Atlas and Bartha 1992). Testing bioremediation was held for the first time in 1989 (Exxon Valdez incident), where 40.9 million gallons of crude oil polluted the 2200 km coastline of Prince William Sound, Alaska. Fined $ 900 million, Exxon Trust contacted the Environmental Protection Agency (EPA) to find an immediate and effective solution. Noting the presence of the taxonomic varieties rich in microorganisms and good aeration of the soil polluted conditions, the researchers decided to use the method of bioremediation by adding nutrients (nitrogen, phosphorus) to increase the rate of biodegradability (Fig. 5.1). Decontamination was a success and was done in record time (Harvey et al. 1990).

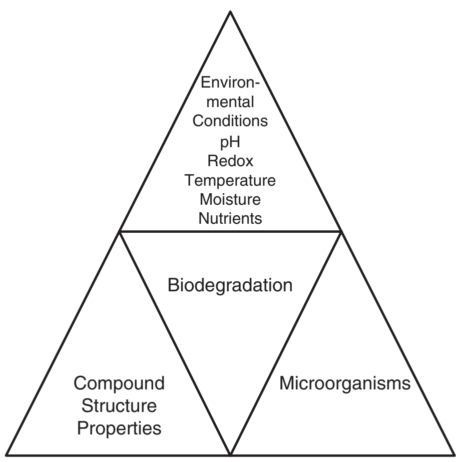

Fig. 5.1 Bioredegradation triangle, Suthersan (1999)

5.2 Bioremediation of Heavy Metals with Microbial Isolates

Microbes are present almost everywhere. They are having adaptations for very low temperatures, extreme heat, and water having varying amount of oxygen along with hazardous components. Energy and carbon sources are the main necessity for microbes (Vidali 2001). Microbes require metal in their life processes. Metals like nickel (Ni), chromium (Cr), copper (Cu), magnesium (Mg), calcium (Ca), manganese (Mn), sodium (Na), and zinc (Zn) are required in many metabolic reactions. Metals like mercury (Hg), cadmium (Cd), gold (Au), aluminum (Al), and silver (Ag) are having no biological role. They are considered as nonessential and are found to be toxic to soil microbes. Microbes from soil may have bioaccumulation of metals 50 times more as compared to normal soil, e.g., *Chlorella vulgaris*, *Oscillatoria* spp. a blue-green algae, and *Chlamydomonas* spp. (green algae). This occurred by two methods:

(i) Direct reduction activity by the bacteria with the use of bioreactors and soils after separating it from main soil. This is called ex situ methods, but it is having limitation of being expensive as well as having low efficiency of metal extraction.
(ii) Indirect method where sulfate-reducing bacteria can be used which produce hydrogen sulfide having capability to precipitate the metals. This in situ method is an environment-friendly, effective, and cheaper approach for treating contaminated groundwater.

With the help of substrates, microbial growth is induced in contaminated soil. During this process the moving metals are immobilized with H_2S due to precipitation reaction produced biologically (Asha and Sandeep 2013). The sulfhydryl group present in tertiary structure of proteins is having capability to bind with these toxic

metals and hence can precipitate them. With the help of in situ bioremediation method, uranium has been removed effectively from soil when it was treated with *Desulfosporosinus* spp. and *Clostridium* spp. (Prasad and Freitas 1999).

5.3 Types of Bioremediation

Bioremediation can be divided into types. (1) In **in situ bioremediation**, treatment of the contaminant soil is done in the same parent location without separating it, which involves direct contacts of microbes with the contaminants being used as substrates for their various biological reactions like biotransformation. But this methodology is time-consuming and cannot be used for quick cleaning of the effected site. (2) In e**x situ bioremediation** which is expensive as well as quick, separate treatment facilities like bioreactors are created in which treatment of contaminated soil is done separately from its parent source (Satinder et al. 2006).

5.3.1 In Situ Bioremediation

This methodology as mentioned earlier is biological in origin used for treating hazardous materials of the soil. This involves the stimulation of growth of naturally occurring bacteria which can degrade organic contaminants by supplying oxygen and nutrients. It can be applied to any contaminated soil and groundwater. It involves induction of water which is having nutrients as well as any electron acceptor like oxygen which leads to the biotransformation reaction. This biotransformation reaction will convert the toxic substance to nontoxic or less toxic compounds having the involvement of modern science technology from many engineering and scientific discipline.

5.3.1.1 Biosparging

A technique named biosparging in which groundwater oxygen concentrations are increased by supplying air through pressure and which is responsible to increase the process of biodegradation through bacteria presents itself in the soil naturally. It simply increases the interaction between soil and water. Due to installation of small air injectors, this technique is considered as cheap and easy to operate, which are the advantages of this methodology.

5.3.1.2 Bioventing

Aerobically degradable contaminants can be degraded by giving extra inputs of oxygen to the existing soil microbes in bioventing methodology. In these techniques to support microbial activity, appropriate oxygen is given by controlling the airflow rates. With the help of air injector, oxygen is given in the contaminated soil. Adsorbed fuel residues which are biodegradable and volatile compounds can be biodegraded from these techniques.

5.3.1.3 Bioaugmentation

In the technique of bioaugmentation, a group of microbes which may be natural or genetically engineered is introduced in the contaminant soil and water for bioremediation. It is the best methodology which is generally used in municipal corporation for treating water. It is fully research-oriented approach which uses genetically improved strains of microbes which may have very high efficiency. In soil and groundwater contaminated with tetrachloroethylene and trichloroethylene, this technology is used where microbes can fully mortify these compounds to chloride and ethylene which are nontoxic. Monitor is one of the limitations of this methodology which is difficult to do here, and the process is also slow in nature.

5.3.2 Ex Situ Bioremediation

When we are having detachment of tainted soil from its hotspot for treatment of contaminant, it is called ex situ approach. It relies upon the period of the contaminant to be evacuated, so on this premise ex situ bioremediation is grouped into two wide structures, strong stage framework (managing soil heaps) and slurry stage frameworks (strong fluid suspensions, bioreactors).

5.4 Phytoremediation

Higher plants are utilized to treat defilement in soil, water, or silt. Many cases are there from the past where it has been utilized for the treatment of overwhelming metals from soil and water, metropolitan sewage and to kill acidic mine seepage. It is classified into various sorts (Asha and Sandeep 2013).

5.4.1 Phytodegradation

It includes phytotransformation, which implies breaking of the contaminant atoms by the plant itself in non-harmful frame by different enzymatic and metabolic responses. Contingent upon the fixation and synthesis, plant species, and soil conditions, contaminants are having almost no possibility without treatment into nonlethal structures. For this situation, the contaminant may turn into the piece of the organic response displayed in the plant itself, and it might be lost as vapors by transpiration and can be phytoextracted.

5.4.2 Phytovolatilization

It is exceptionally critical system appeared by plants in which plants are equipped for discharging the corrupted type of contaminants might be from stomata of leaves and stems. The non-harmful used type of the contaminate turns into the piece of transpiration pathways and discharged noticeably all around. This has been accounted for if

there should be an occurrence of biodegradation of trichloroethene (TCE) or its breakdown items in poplar plants. One of the reports additionally demonstrates that the changed tobacco plants can take up profoundly lethal methylmercury and change over them in unpredictable frame which is generally sheltered levels of mercury. Once volatilized, it might take part in numerous responses in the environment with hydroxyl radicals and turn into the piece of the photochemical cycle.

5.4.3 Phytostabilization

The poisonous mixes can be immobilized in soil by plant itself which is known as phytostabilization. The foundation of established vegetation anticipates windblown tidy. Vast volume of water that is originating from plants by transpiration can be used as pressurized water control. It can be connected to those territories where contaminant metals can be restricted to a specific zone as a most ideal choice for their bioremediation by keeping their spreading in the dirt and condition. As metals don't at last debase, keeping them in restricted range is the best option with low pollution levels.

5.4.4 Phytoextraction

Plants are having the capacity to take up the organic contaminant by their foundations and send them in over the ground in shoot or clear out. Here one condition is vital that the contaminant under examination is ought to be dissolvable in water with the goal that it can be uptaken by the plants. Once the contaminant is broken up in water, then it can be effectively taken up through plant transport systems.

5.4.5 Rhizofiltration

In this innovation of the bioremediation, the roots introduced in the dirt in polluted areas are in charge of bioremediation. Root is having the ability of hastening the contaminants, primarily the substantial metals from watery arrangements. It is the place huge in those regions where wetland can be made and all the polluted water is permitted to move in that wetland to come in coordinate contact with the roots. Underlying foundations of plants have been examined for bioremediation against huge amounts of lead and chromium from soil water.

5.5 System of Microbial Remediation

Microorganisms interface with various substantial metals with various methodologies. Imperviousness to metal is the primary instrument of substantial metal remediation. It is discovered that substantial metal conflict microorganisms may be developed in light of overwhelming metal introduction. It is likewise feasible for

microorganisms to contain autonomous resistance components that don't require metal worry for actuation. The procedures depending on organisms can be partitioned into three sorts: first one is biosorption (bioaccumulation) in which microorganisms think and incorporate contaminants of metal on its cell structure (Maier et al. 2009); in second, procedure of extracellular precipitation and take-up by purged biopolymers (Chu et al. 2010); and the third may incorporate the help by a particular atoms got from microbial cells (Maier and Soberón-Chávez 2000).

Biosorption is considered as an essential process and biologically it is a useful term. Materials can immobilize which present on the external surface area of cell, the metal through anionic cell surface useful in gatherings which contain substantial cationic metals including Pb, Fe, Zn, and Cd. Normally the coupling can be proficient with sludge of layers consisting of starches, nucleic, polysaccharides, and unsaturated fats (Maier et al. 2009). Dynamic practical gatherings of extracellular restricting materials assume imperative part in the biosorption procedure. Metal particles bound to cell surfaces by means of a scope of restricting systems including electrostatic collaborations, van der Waals powers, covalent holding, redox connections, and extracellular precipitation (Blanco 2000). Practical gatherings in an enacted state such as amine bunches in peptidoglycosides, acetamido bunches in chitin, carboxyl and sulfhydral gatherings in protein, phosphodiester, and hydroxyl and phosphate groups in polysaccharides participate during biosorption procedure (Rajendran et al. 2003). Microscopic organisms are superb biosorbents because of a large surface area proportions and a decent type of possibly dynamic chemosorption locales, e.g., teichoic corrosive in a bacterial cell divider (Beveridge 1989).

Another component is intervened during siderophore arrangement. Siderophore is small subatomic weight or mass-chelating operators delivered by microscopic organisms, parasites, and plants to encourage a take-up of Fe (iron) (Chu et al. 2010). Alongside their ability to encourage microorganisms with press, siderophores can likewise chelate different metals too. Metals except iron can fortify a creation of siderophores by microscopic organisms, in this manner ensnaring siderophore during homeostasis of metals except press and particularly substantial metal resistance (Schalk et al. 2011). Cooperation of siderophores with different metals having science like that of iron, for example, Al, Ga, and Cr, shapes trivalent particles comparative in size to press. In this way, siderophores, by restricting overwhelming metals, can decrease both bioavailability and metal poisonous quality; e.g., siderophore-interceded association diminishes copper danger in cyanobacteria (Stone and Timmer 1975), and in *Alcaligenes eutrophus* and *Pseudomonas aeruginosa*, siderophore amalgamation is instigated by overwhelming metals within sight of iron fixations (Höfte et al. 1994).

Creation and discharge of biosurfactants from microbes may upgrade bioremediation of substantial metals. Biosurfactant atoms can shape buildings with metals, for example, Pb, Cd, and Zn (Maier and Soberón-Chávez 2000). The anionic nature of biosurfactants can catch metal particles through electrostatic communications (Rufino et al. 2012). Complexations shaped by biosurfactant atoms increment the evident solvency of metals. In this way, metal bioavailability can be affected by

normal metabolic side effects that outcome in metal lessening bringing about the arrangement of less dissolvable metal salts including sulfide and phosphate encourages (Maier et al. 2009).

5.5.1 Cadmium (Cd)

Cadmium and its subsidiaries are very versatile in soil; these are more bioavailable and have a tendency to bioaccumulate because of their higher relative solvency. Disc is maybe the most mindfully followed because of potential poisonous quality to people and its relative portability in soil-plant frameworks (Tran and Popova 2013). The biggest wellspring of air Cd discharges is metal creation, trailed by squander burning and other minor sources incorporate generation of batteries consisting nickel-cadmium, nonrenewable energy source ignition, and modern tidy era. Water bodies are for the most part defiled by Cd through handled water sources from phosphate mining, smelter and related compost generation, and electroplating squanders. The significant course of Cd when it enters into the body of human being is intake, particularly of plant-based foodstuffs (World Bank Group 1998). Applying harmfulness basically to renal system, Cd can cause demineralization of bones and may weaken lung capacity and increment the danger of lung disease (Bernard 2008). For example, during the 1950s, Cd pollution prompted kidney disability and bone ailment (Itai-itai infection) in uncovered populaces in Japan (Kaji 2012).

Imperviousness to Cd in microbes depends onto Cd transition. Metallothionein proteins present in *Cyanobacteria* and metallothionein smt locus build the Cd (cadmium) confrontation, and its erasure diminishes resistance (Gupta et al. 1993). Cadmium is by all accounts detoxification by (gram $^{-}$ve) microscopic organisms with assistance of RND (Resistance Nodulation Cell Division) frameworks like czc, which is principally a zinc exporter (Schmidt and Schlegel 1994). CorA and NRAMP (Natural Resistance Associated Macrophage Protein) help the Cd^{2+} to enter the gram-negative bacterial cell by – like take-up frameworks, ties to thiol mixes, applies poisonous quality and is traded again by CBA (cytometric bead array), P-sort ATPases, and CDF (cation diffusion facilitation) proteins (Nies 2003). In gram-positive microscopic organisms, this happens by RND-driven trans-envelope and perhaps at the same time by CDF transporters (Nies 1999). In *S. cerevisiae*, glutathione ties cadmium and transportation of secondary cadmium biglutathionate complex by ABC transporters and YCF1P into vacuole (Li et al. 1997).

5.5.2 Chromium (Cr)

Anthropogenic sullying of chromium (Cr^{6+}) is due to broad applications in different enterprises, for example, steel generation, electroplating of chrome, colors, calfskin tanning, and wood additives (Das and Mushra 2008), and its high solvency and poisonous quality make its remediation a major need that has been plentiful at close dangerous levels since the source of life (Mukhopadhyay et al. 2002).

5.5.3 Arsenic (As)

Arsenic introduction into the human body may happen by nourishment, water and air; water is a significant course of presentation and every main interminable, As harmful originated to water (Kapaj et al. 2006). Bangladesh is an illustration where As pollution is exceptionally normal and followed the history of arsenicosis patients. It has been assessed that 57 million people groups in Bangladesh are encountering introduction to As in their drinking water (Appelo 2006). The significant well-being dangers of arsenic (As) poisonous quality are keratosis or hyperpigmentation prompting an expanded the dermatological problems, effect on growth vital organs (Kapaj et al. 2006). Arsenate copies phosphate when entering the microbial cell by means of carriers, from that point meddling with phosphate-based vitality creating forms and at last repressing oxidative phosphorylation. Glycerolporins (a noteworthy layer channel family protein) focuses on a more extensive scope of cell forms, authoritative to thiol bunches in essential cell proteins, for example, 2-oxo-glutarate dehydrogenase and pyruvate dehydrogenase (Lloyd 2005).

Microorganisms have the capacity to utilize methylation like detoxification process for remediation of arsenic from neighborhood condition. For instance, the process of methylation growths may deliver monomethyl arsonic (MMA) corrosive or dimethyl arsenic (DMA) corrosive, and unstable methylated arsines are produced by prokaryotes. The arsenate reductase protein plays a significant role in remediation of arsenic by microscopic organisms and yeasts. For the detoxification of arsenic, qualities of ArsC and different proteins required are encoded regularly on plasmids. Approximately 100 arsenic operons have been rearranged or sequenced (Mukhopadhyay), and this arrangement will be essentially large at this point.

5.5.4 Lead (Pb)

The lethal idea of lead is perceived for centuries, by the most punctual distributed information going back to 2000 BC (Needleman 1999). Pb has broadened applications in fuel oil, paints, earthenware production, nourishment jars, makeup, batteries, and so forth. So it is available in air, clean water, and soil to fluctuating degrees with human presentation happening through ingestion, inward breath, and dermal retention (Ezzati et al. 2004). Pb is a total toxicant that influences hematological, neurological, cardiovascular, gastrointestinal, and kidney frameworks of human body. It is assessed that 0.6% of the worldwide weight of malady, with most noteworthy weight in creating locale, is represented by Pb presentation.

Lead does not make some phenomenal lethality from microorganisms which amass Pb_2^+ through biosorption forms. Importation of Pb_2^+ into microorganisms happens through take-up frameworks which have a place with different protein groups of divalent transportation of cationic metal P-sort ATPases enzyme, while send out is intervened with the help of ATP-hydrolyzing efflux frameworks. The pbr operon contains parts of this type of arrangement for microorganism resistant to lead (Tsai et al. 2002).

5.5.5 Copper (Cu)

The copper creation through the world is rising day by day, prompting increasingly copper in the earth. Utilization of Cu incorporates as a segment in electrical hardware, development, modern apparatus, and manures. Copper ordinarily drains into sources of water from Cu funnels and from added substances intended to regulate algal development. Copper is a fundamental metal for organic frameworks. Copper firmly builds with natural materials in dirt, inferring that a lone little part of copper will be found in arrangement as ionic copper, Cu (II). But in introduction to high measurements, Cu does not make lethality. Be that as it may, long haul introduction to Cu can cause iron deficiency, disturbance in major or vital organs like the kidney and liver and stomach and intestine (Wuana and Okieimen 2011).

Cu harmfulness depends on its radical nature prompting hyperoxide particle generation which interfaces with cell layer through official with thiol mixes (Nies 2003). In gram-positive microorganisms, P-sort ATPases appear to detoxify Cu through efflux. In a few microorganisms, Cu conflict proteins encode which tie Cu present in the periplasm or near external film (Nies 1999).

5.5.6 Zinc (Zn)

Zn can be found in extensive amounts in water and soil as the world's Zn generation keeps on rising. Zn can bio-amplify up to the evolved way of life in water bodies or soil. It is likewise critical to take note of that lone a set number of plants have a shot of survival in Zn-rich soil. Intense lethality to people by zinc emerges from the ingestion of over-the-top measure of zinc salts either by chance or intentionally as an emetic or dietary supplement.

5.5.7 Cobalt (Co)

Co (cobalt) danger is very low contrasted with numerous different metals present in soil. Cobalt is available in environment as a metal and in Co (II) and Co (III), i.e., two valence states which frame different natural and inorganic compounds and salts. Metal cobalt is working as cofactors for a few compounds for organic frameworks and is likewise critical for amalgamation of cobalamine or vitamin B_{12}. On the other hand, Co can add to unfavorable well-being impacts on lungs, including pneumonia, asthma, and wheezing when introduction happens in abnormal states.

5.5.8 Nickel (Ni)

Nickel metal may discover its mode into human body in a roundabout way, e.g., during sustenance which has been taken care of, utensils using for handling and cooking containing expansive amounts of nickel. Despite the fact that Ni and Ni mixes with exemplary harmful specialists acknowledge in industries, all general

society might be accessible to nickel visible all around, water and nourishment. The toxic quality and cancer-causing nature of some Ni mixes in exploratory creatures and in occupationally exposed general population are all around reported (Cempel and Nikel 2006). For example, Ni-carbonyl is generally intense poisonous nickel compound that can cause frontal migraine, queasiness, regurgitating, sleep deprivation, and touchiness in its quick harmful impact. A moment auxiliary quality locale, cnr, which depends on cationic efflux as a conflict determinant is made out of cnrCBA basic area (Nies and Silver 1989; Sensfuss and Schlegel 1988; Liesegang et al. 1993) went before by an administrative quality district. Another Nikel and cobalt resistance determinant is ncc and was likewise portrayed (Schmidt and Schlegel 1994). Like cnr, ncc is made out of an administrative quality area took after by the basic locale nccCBA.

5.6 Bioremediation Research Studies on Bioremediation Research in Developed Laboratory Bioreactors

5.6.1 Bioremediation of Pesticide: For Soil Treatment by Utilizing Microbial Consortia

Utilization of pesticides has been expanded massively in India. The waste items from pesticide business have turned into a natural issue because of inadequate and insufficient waste treatment innovation. The accessible data demonstrates that pesticide buildups stay in surface soil, prompting harmfulness in dirty water condition. Current achievement in bioremediation innovation utilizing microbes has been discovered viable for treatment of soil from harmful pesticides. A unit for the treatment of surface soil was composed wherein bioremediation of usually utilized pesticides to be specific cypermethrin, chlorpyrifos triclopyr butoxyethyl ester, and fenvalerate, at different fixation, had been completed effectively utilizing dairy animals waste microbial consortia under recreated natural conditions. The conditions such as bioremediation has been observed and kept up along with the examination. The examination was reached out till the parentage of compound was changed over intermediates as well as unsafe mixes. The outcomes demonstrated the capability of dairy animal compost slurry consortia for bioremediation of soil debased with pesticides in treatment of surface soil (Fulekar and Geetha 2008).

5.6.2 Utilization of Bioreactors for Bioremediation of Pesticides

To appraise the process of bioremediation capability of *Pseudomonas aeruginosa* by enhancing its flexibility toward expanding centralization of chlorpyrifos utilizing bioreactors, *Pseudomonas aeruginosa* seclude NCIM 2074 was adjusted by subjecting to shifting groupings of chlorpyrifos. An underlying convergence of chlorpyrifos was provided in insignificant salt medium (MSM) under controlled natural conditions. The way of life was consequently scaled up to elevated centralizations

of chlorpyrifos. This procedure was rehashed, every time utilizing medium with elevated chlorpyrifos focus. The whole scale-up process proceeded for a time of 70 days. *Pseudomonas aeruginosa* has potential use in bioremediation of chlorpyrifos; however the living being is repressed by higher fixations (Fulekar and Geetha 2008).

5.6.3 Bioremediation of Benzene by Utilizing a Bioreactor

A bioreactor was created for parcelling of watery and natural stages with an arrangement for air circulation and blending, a cooling framework, and an inspecting port. The capability of a dairy animal's waste microbial consortium was surveyed for bioremediation of phenol in a solitary stage bioreactor and a two-stage parcelling bioreactor. The *Pseudomonas putida* IFO 14671 was detached, refined, and distinguished from the cow excrement microbial consortium as a high-potential phenol degrader. This displays a propel strategy in bioremediation systems for the biodegradation of natural compound, for example, phenol utilizing a bioreactor (Fulekar et al. 2009).

5.6.4 Utilization of Dairy Animals Manure Microflora in Two-Stage Parcelling Bioreactor for Bioremediation of Benzene

For bioremediation of benzene bovine manure, microflora has been utilize in a bioreactor. The benzene bioremediation is affected by cow compost microflora which was observed inhibitory for benzene. Thus to carry out biodegradation, two-stage parcelling bioreactor (TPPB) has been created at higher focus. Assistance of the *Pseudomonas putida* MHF 7109 was disconnected from bovine manure microflora as strong degrader of benzene, and its capacity to corrupt benzene at different focuses has been assessed. The GC-MS information likewise demonstrates the nearness of 2-hydroxymuconic semialdehyde and catechol, which affirms built-up pathway of benzene biodegradation. This demonstrates capability of dairy animal's fertilizer microflora as a wellspring of biomass for degradation of benzene in TPPB (Fulekar et al. 2009).

5.6.5 Utilization of Ryegrass for Bioremediation of Pesticide Chlorpyrifos in Mycorrhizosphere Natural Remediation

Rhizosphere bioremediation of chlorpyrifos in mycorrhizal soil has been explored by the greenhouse pot culture tests. The pot-refined soil corrected at introductory chlorpyrifos grouping was seen to be debased where revised fixations diminished remain quickly affected by ryegrass mycorrhizosphere as the brooding. In soil, the microorganism ascribed bioremediation of chlorpyrifos these microorganisms related with ryegrass rhizosphere, along these lines, microorganisms making due in

rhizospheric soil spiked at most noteworthy focus has been surveyed and utilized for confinement of chlorpyrifos debasing microorganisms. 16S rDNA examination utilizing BLAST method to distinguish potential degrader was *Pseudomonas nitroreducens* PS-2. The heterotrophic microscopic organisms and parasites were additionally identified from the immunized and non-vaccinated rhizospheric soils.

5.6.6 Utilization of *Pseudomonas putida* Strain MHF 7109 for Biodegradation of Oil Hydrocarbon Mixes Toluene and O-Xylene

Pseudomonas putida strain MHF 7109 has been separated and distinguished from dairy animal's waste microbial consortium for biodegradation of chosen oil hydrocarbon mixes – toluene, benzene, and o-xylene. Every compound was connected independently in negligible salt medium to assess debasement movement of the distinguished microbial strain. The outcomes showed that the strain utilized can possibly corrupt BTX at a centralization of toluene, and benzene was observed to be totally debased individually. It has been found at higher focuses that BTX repressed the movement of microorganisms. *P. putida* MHF 7109 has high potential for biodegradation of unstable oil hydrocarbons (Singh and Fulekar 2010).

5.7 Hereditary Engineering

Many hereditarily designed microorganisms have been utilized to expand their capacity to process particular chemicals, for example, hydrocarbons and pesticides as their wellspring of vitality. In the 1980s hereditary designing for development of bioremediation process was on blast. These strategies are considered to improve the debasement of unsafe waste products under facility of lab research. They have advanced bioremediation edge and shown efficiently for the corruption of altered contaminations under considered conditions. Hereditary alteration innovation has important applications for use during the time spent bioremediation. Bioremediation investigates quality-assorted variety and metabolic flexibility of microorganisms (Fulekar et al. 2009). The hereditary cosmetics of these living beings make them significant in biosorption biodegradation, bioaccumulation, and biotransformation. The feature encoding for compounds in charge of biodegradation is available in chromosomal and additional chromosomal DNA of these microorganisms. Recombinant DNA procedures encourage to advance the capacity of a life form to use a xenobiotic by discovery of such degradative qualities and changing them into fitting host by means of appropriate vector. It relies upon weakness of modification and trade of hereditary data. The different methodologies connected in recombinant DNA innovation are PCR, hostile to detect RNA strategy, site-coordinated mutagenesis, electroporation, and molecule assault procedures. The advanced life science with recombinant DNA innovation is presently capable bioremediation innovation for enhancing pollutant-debasing organisms. Hereditary alteration of

particular administrative and metabolic qualities that are critical for creating viable, sheltered, and sparing systems for bioremediation is of incredible significance. Bioremediation is not powerful just for debasement of poisons except it can be utilized to sanitary undesirable substances from soil water and air.

5.8 Biomarkers for Checking Productivity of Bioremediation

Distinctive biomarkers are accessible as instruments for labeling microscopic organisms. The decision of biomarker relies upon the framework examined and the pretended by this (Jansson 1995).

5.8.1 Luciferase Biomarkers

Luciferases as biomarkers are helpful for observing the bioremediation inocula. Microscopic organisms labeled with the firefly luciferase quality, or bacterial luciferase qualities (luxAB), can be effectively identified, so it can be considered as luminescent provinces on plate of agar gel. For instance, a biosurfactant delivering luxAB-labeled strain of *Pseudomonas aeruginosa* was followed by including radiant settlement oil-debased soil microbes (Flemming et al. 1994). The strain of *P. aeruginosa* was additionally labeled with lacZY qualities, lactose permease, and encoding b-galactosidase individually, by giving blue state development on X-lady contain in medium (Flemming et al. 1994).

The principal advantage is the capacity to straightforwardly screen light yield, exclusively the need for development of the cells. The yield of light is demonstrative of cells which are metabolically dynamic (Rattray et al. 1990). In the event that the cells are developing, the yield of light is relative for quantity of cells in this case. In any case, after long haul brooding in soil conditions, or other "harsh" situations, microbial cells frequently wind up plainly starved or pushed, and the light generation a reaction from luciferase compounds decays to the change in status of cell vitality (Duncan et al. 1994). It demonstrates that in situ bioluminescence is an unstable marker of microbial biomass under some conditions like starvation. An individual strategy to defeat this is to point the specimen with supplements to actuate the microbial populace (Meikle et al. 1992; Duncan et al. 1994).

Another approach is immediate extraction of aggregate protein from the ecological example, which incorporates the luciferase protein. At that point, the addition of vitality sources straightforwardly to the protein removes in vitro, and the luciferase chemical movement can be associated to the particular luciferase-labeled microbial biomass in the specimen (MoÈ ller et al. 1995; MoÈ ller and Jansson 1998). These sorts of estimations have been utilized to quantitate microorganisms used to bioremediate fuel or chlorophenol contaminants in soil.

5.8.2 Utilization of the Luc Quality as a Biomarker for Checking a Fuel Corrupting *Pseudomonas*

The quality luc was utilized to mark a fuel corrupting *Pseudomonas*. A smaller than usual, conveying that the luc quality was combined with tac promoter (MoÈ ller and Jansson 1998), this transposon vector (pAM103) was utilized to embed the luc quality into chromosome of *Pseudomonas fluorescens* 935061, the fuel corrupting segregate. An individual mutant, assigned 940022, had most astounding beam yield by a factor but, however, had disabled development when contrasted with the wild sort strain, while another mutant, strain 940030, was decided for later examinations since it had an elevated light yield yet kept on developing likewise to the wild sort strain. Gas having inhibitory impact on the development of microscopic organisms is outstanding (Sikkema et al. 1995). It is because of changes in the film because of the associations of lipophilic solutes with various parts of the layer. To check harmfulness of fuel against luciferase action, TGY 1/5 developed cells from inactive stage societies were presented to various centralizations of gas, and the luciferase movement was resolved. Gas has no noteworthy impact on luciferase action in the range tried. The way of life was routinely developed in TGY1/5 medium, since the way of life was developed in present medium with a high light production on an optical thickness premise.

Survival of the luc-labeled gas debasing microorganisms was evaluated in soil microcosms. The dirt began from a similar area of gas station for *P. fluorescens* strain 935061 had been separated. Luciferase movement was considered in immunized soil microbes. During test of light creation in soil, the separation of cells from the dirt following strategies beforehand was portrayed (MoÈ ller et al. 1995; MoÈ ller and Jansson 1998). The extricated cell lyses by solidifying into fluid nitrogen, lysozyme treatment, and defrosting. The protein extricates (counting luciferase) were then focused by channel centrifugation before expansion of business support, including ATP and luciferin substrate, as beforehand depicted (MoÈ ller et al. 1995; MoÈ ller and Jansson 1998), and tests were measured for 5 s utilizing a BioOrbit 1253 luminometer. The underlying luciferase movement was around five overlays higher in microcosms vaccinated with cells developed in TGY 1/5 medium than in the microcosms immunized with cells developed in negligible medium with fuel as a carbon source. The luciferase action diminished to underneath the location level following 21 days of hatching. The decrease was most fast for the TGY1/5 developed cells. No luciferase movement was recognized in the control microcosms, featuring the specificity of this marker. Also, the luciferase action estimations were better than plate meaning following of the *Pseudomonas*, because of poor development of states on insignificant medium and because of foundation development of the indigenous microflora on wealthier medium.

5.8.3 Utilization of the Luc Quality as a Biomarker for Observing a 4-Chlorophenol Corruption

A 4-chlorophenol-corrupting strain of *Arthrobacter* (Westerberg et al. 1999) was chromosomally labeled with the luc quality, utilizing the pAM103 vector (MoÈ ller and Jansson 1998). Despite the fact that the pAM103 vector has the administrative quality, lacI, in the development, there was no huge increment in light endless supply of IPTG to the medium. It demonstrated the enormous articulation of the luc quality from the tac promoter in these cells. In any case, the luciferase action measured in vitro (cell extricates) was brought down in *Arthrobacter* cells than in the *P. fluorescens* cells, accepting the *Arthrobacter* cells were more difficult to lyse. These cells pre become under these conditions were then vaccinated into soil. Gas-corrupting *Pseudomonads* survived better when pre-grown in rich medium. These distinctions are because of various components in catabolic quality enlistment or contrasts in stretch incited survival instruments in the two strains. The luc-labeled *Arthrobacter* was observed in various soil sorts by assurance of luciferase action in protein removes (in vitro).

5.8.4 GFP as a Biomarker

One powerful marker for observing the bioremediation is the gfp quality, which encodes for GFP, green fluorescent protein. The benefit of GFP is that the protein fluoresces upon brightening with blue light and no other vitality source or substrate expansion is required, other than oxygen amid introductory arrangement of the chromophore. The GFP quality has been improved as a marker for microscopic organisms in ecological examples (Unge et al. 1997a; Tombolini and Jansson 1998).

5.8.4.1 Utilization of GFP as a Biomarker for Checking a 4-Chlorophenol-Debasing *Arthrobacter* Strain

The 4-chlorophenol-corrupting strain of *Arthrobacter* was labeled with two duplicates of gfp quality (Unge et al. 1997b). A particular fluorescing GFP *Arthrobacter* cells was envisioned in soil tests by epifluorescence microscopy. Be that as it may, albeit many transformants were broken down, cells labeled with two gfp duplicates were not fit for debasement of 4-chlorophenol without expansion of LB medium. By differentiate, when the *Arthrobacter* cells were labeled with a solitary duplicate of gfp, the cells were similarly efficient at 4-chlorophenol debasement contrasted with the wild sort (ElvaÈ ng et al. 1999). It is an unidentified purpose behind inappropriateness of two gfp duplicates and 4-chlorophenol debasement in *Arthrobacter* cells; however, it might be because of poisonous quality impacts.

5.8.4.2 Utilization of GFP as a Biomarker for Observing Bioremediation Inocula

Numerous cases of gfp utilization as a biomarker to observe bioremediation inoculate have been distributed. For instance, a strain of *Moraxella* as P-nitrophenol corrupting (Tresse et al. 1998) and a mineralized strain phenanthrene of *Pseudomonas* (Errampalli et al. 1998) that is followed in soil microcosms by checking of GFP fluorescent states. Culture-free strategies have additionally been effectively shown for count of GFP-labeled cells, for instance, by specification of fluorescent cells utilizing a stream cytometer (Tombolini et al. 1997).

5.9 Points of Interest of Bioremediation

- It is a characteristic procedure for squander treatment from defiled material, for example, soil. Organism number may increment or diminish as per the measure of the contaminant.
- The deposits left after the treatment are typically innocuous items.
- Bioremediation requires a less exertion and can be done nearby, without interruption of ordinary exercises.
- This likewise decreases dangers to human well-being and the condition that can emerge amid transportation.
- Bioremediation is additionally a financially savvy process as it costs not as much as the other regular techniques.
- It does not utilize any unsafe chemicals. The supplements included for the development of microorganisms are ordinary composts which are normally utilized, not any unsafe chemicals.
- Since bioremediation changes the toxins into water and innocuous gasses, consequently contaminations are totally wrecked.

5.10 Impediments of Bioremediation

- It is restricted to biodegradable materials. It can't be connected to all mixes.
- There is additionally some worry that mixes might be more persevering contrast with parent at least one lethal after biodegradation.
- Level of specificity is high in biodegradation which make it all the more exorbitant a few times.
- Application of seat- and pilot-scale concentrates to full-scale field operations is frequently troublesome.
- New bioremediation advancements must be hunts that are proper down destinations with complex blends of contaminants.
- Bioremediation forms are regularly longer than other treatment choices, for example, unearthing and expulsion of soil or burning.

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6 Bioremediation: An Eco-sustainable Approach for Restoration of Contaminated Sites

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Abstract

In the current scenario, pollution of soil, surface water, and groundwater, with toxic chemicals due to industrialization, is one of the global concerns for the sustainable development of human beings. Thus, the eradication of toxic organic and inorganic pollutants from the contaminated environment is the need of global concern to advance the sustainable development with low environmental impact. The treatment of contaminated soil, sediment, and water by the conventional method is found to be unfeasible due to its high cost and generates secondary pollutants. Therefore, bioremediation has emerged as a natural, economic, sustainable approach which can restore the contaminated soil, surface water, and groundwater, with the help of biological agents like bacteria, fungi, and other organisms or their enzymes. It is an evolving green technology where microbes are grown in the presence of contaminated soil, sediment, surface, and groundwater to elevate the decomposition and/or removal of inorganic and organic pollutants. Bioremediation technologies can be broadly categorized into two categories, i.e., in situ bioremediation and ex situ bioremediation. In situ bioremediation involves treatment of contaminated substances at the same place, whereas ex situ bioremediation involves the elimination of the contaminated material which is treated somewhere else. Some typical examples of bioremediation technologies involve bioventing, biosparging, bioaugmentation, land farming, composting, and biostimulation. This book chapter gives a gist about bioremediation, its strategies, factors affecting biodegradation processes, and advantages and disadvantages of bioremediation.

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6.1 Introduction

With the increasing human activities like in agriculture, industries, and urbanization over the last decades, a broad range of anthropogenic chemicals have been introduced into the water, soil, and air, which has caused extensive environmental problems. These hazardous chemicals comprise an array of organic compounds like polycyclic aromatic hydrocarbons, petroleum hydrocarbons, xenobiotic compounds, halogenated hydrocarbons, phenolic compounds, volatile organic compounds (VOCs), nitroaromatic compounds, polychlorinated biphenyls (PCBs) and pesticides, and inorganic compounds such as nitrate, phosphates, salt, and heavy metals, i.e., arsenic (As), copper (Cu), zinc (Zn), mercury (Hg), lead (Pb), cadmium (Cd), chromium (Cr), nickel (Ni), selenium (Se), and silver (Ag). Contaminated ecosystems adversely affect the growth and metabolic activities of the soil microbes, soil structure and fertility, plants, aquatic organisms, and biogeochemical cycling of elements which finally affects the ecosystem along with the human health. Thus, the eradication of organic and inorganic pollutants from the contaminated area is the firm requirement to endorse a sustainable development of our society. A wide series of chemical and physical methods (i.e., soil washing, land filling, soil washing, excavation, incineration, adsorption, coagulation, flocculation, filtration, photodegradation, and chemical oxidation) are used for eradicating organic and inorganic pollutants which are not only time-consuming and expensive, but also they do not offer a complete solution. Curiosity to explore microbial biodegradation of toxic contaminants has exaggerated in recent years as mankind attempts to attain sustainable approach for cleaning up and restoration of contaminated environments. Bioremediation is a cost-effective, sustainable, and natural approach (compared to other traditional technique) to clean up the contaminated soil, sediments, and water with the help of the naturally occurring organisms such as fungi, bacteria, or their enzymes (USEPA 2006; 2012). It is a desirable waste management technique which offers partial decontamination, maintenance of biological activity, physical structure and microbes of soils, and site restoration. In bioremediation technology where microbes are cultured in the presence of hazardous contaminates in order to enhance the decomposition and/or removal of inorganic and organic pollutants. This book chapter aims to provide an overview about bioremediation, the focus is on microbial processes since they play a significant role in the degradation, i.e., cycling of organic compounds in the environment. In addition, the objective of this chapter is to provide an abridged discussion about the processes allied with the use of bioremediation as a cleanup method for the remediation of the hazardous industrial waste from

the contaminated site. In this chapter, we depicted various processes of bioremediation including in situ and ex situ remediation technique with particular emphasis on plant-assisted bioremediation remediation of organic and inorganic pollutants. Further, we have also discussed the challenges of bioremediation technique for elimination of toxic pollutants from the contaminated site.

6.2 Bioremediation and Its Strategies

Bioremediation is an eco-friendly method which employs many different microbes, works in parallel or series of the sequence to vitiate and/or detoxify toxic contaminants. In other words, it can be stated as the speeding up of the normal metabolic process, whereas microorganisms (i.e., bacteria and fungi), green plant (termed phytoremediation), or their enzymes disintegrate or transform toxic contaminants into CO_2 (carbon dioxide), H_2O (water), microbial biomass, inorganic salts, and other by-products (metabolites) which are less toxic than the parental compounds (Chakraborty et al. 2012). Thus, by employing the microbes for degradation and detoxification of pollutants is now being increasingly applied as the technology of preference for clean up or to restore contaminated sites back to a sustainable environment (Megharaj et al. 2011). Considering the transportation and removal of pollutants from contaminated sites, bioremediation technology can be grouped into two categories: (1) in situ bioremediation and (2) ex situ bioremediation (Fig. 6.1).

6.2.1 In Situ Bioremediation

In situ bioremediation is the process which is performed at the original site of the contamination (USEPA 2006, 2012). In situ bioremediation is primarily used to treat contaminations persisting in the saturated soil and groundwater. In this technique, oxygen (O_2) and nutrients (mostly carbon and nitrogen sources) are added to the contaminated site or the environment in order to accelerate the growth of microbes and escalate the rate of biodegradation. The microbes may be indigenous; however, the microbes which are highly effective in degrading the pollutants may be acquainted at the site. During in situ bioremediation processes, chemotaxis, the microbial movement toward or away from chemicals, plays a substantial role because microbes having chemotactic abilities can move toward the contaminated area and boost the in situ degradation of pollutants. This in situ bioremediation is further subdivided into two broad types: (1) intrinsic in situ bioremediation and (2) engineered in situ bioremediation (Hazen 2010) (Fig.6.1).

6.2.1.1 Intrinsic In Situ Bioremediation

Intrinsic in situ bioremediation (natural attenuation (NA) or passive bioremediation) is a degradation process of organic compounds persisting in the contaminated soil

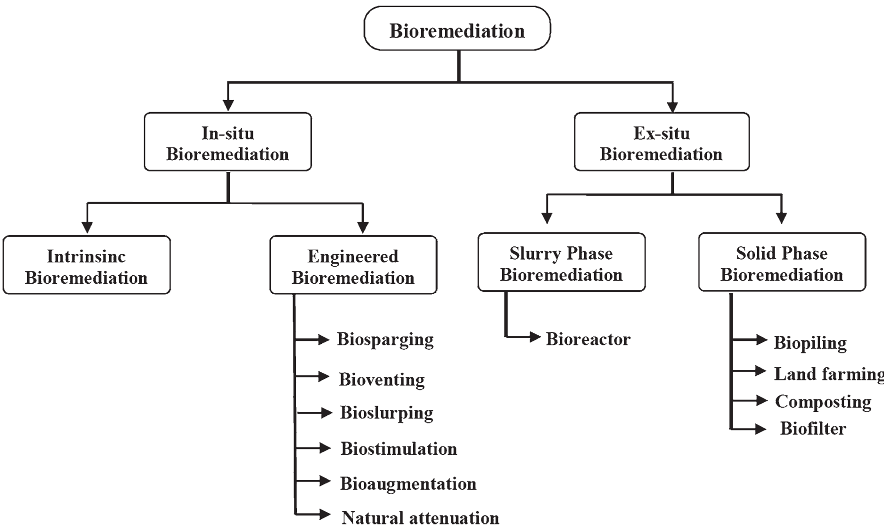

Fig. 6.1 Different strategies of bioremediation

by naturally occurring (indigenous) microbes, without any artificial augmentation. Intrinsic bioremediation depends on the underlying metabolic activities of indigenous microbes to degrade and/or transform hazardous pollutants without utilizing any artificial steps to enhance the degradation process (USEPA 2000a, b, 2006). Indigenous microorganisms are a assembly of native microbial group that inhabits the soil, industrial sludge, and the surfaces of all living things either it be inside and outside which have the prospective in biodegradation, improving soil fertility, phosphate solubilizers, nitrogen fixation, and plant growth promoters. There are four primary needs that must be met for intrinsic in situ bioremediation to be successful. These four requirements are an (i) adequate populace of biodegrading microbes at the contaminated site, (ii) adequate nutrients should be available for microbial growth, (iii) ideal environmental conditions (i.e., pH, temperature, oxygen) prevail at the contaminated site, and (iv) proper time should be given to microbes natural process to deplete the contaminant. Intrinsic bioremediation may play the role of MNA (monitored natural attenuation) sites. MNA can be described as biodegradation, dilution, dispersion, volatilization, sorption, radioactive decay, and biological chemical or stabilization or transformation of pollutants (NRC 2000; USEPA 2000a, b). This suggests that hazardous pollutants are left in place, while NA works on them. The term NA is employed for all naturally occurring processes that are accountable for the remediation of hazardous pollutants at the contaminated site. The USEPA (US Environmental Protection Agency) defines NA is a remediation approach include a variety of physical, chemical, or biological processes that, under ideal conditions, to lower the toxicity, mass, volume, mobility, or concentration of pollutants in soil, sediment, or groundwater, without any human interference. These in situ processes include volatilization, dispersion, sorption, biodegradation, dilution, and chemical or biological stabilization, destruction or transformation, of

contaminants (USEPA 1999). To augment the NA, microbial activity can be reinforced (stimulated) by optimizing the environmental conditions, i.e., oxygen or other electron acceptor accessibility, nutrient content, temperature, pH, and redox conditions.

6.2.1.2 Engineered In Situ Bioremediation

The engineered in situ bioremediation also known as accelerated in situ bioremediation, the introduction of indigenous microbes to contaminated site, accelerates the biodegradation process by developing or enhancing conductive physicochemical conditions of an environment (Hazen 2010). In engineered bioremediation, nitrogen (N), oxygen (O), and phosphorus (P) are disseminated through the subsurface via an instilment or extraction well in order to stimulate the growth and metabolism of existing microbes. Microbes using O_2 as an electron acceptor convert it to H_2O as they disintegrate the toxic pollutants. When contaminated site conditions get inauspicious, engineered bioremediations are introduced to the contaminated site, predominantly with genetically altered bacteria. It is presumed that free-living genetically altered bacteria may have less chances of survival due to the stress conditions imposed by the introduction of foreign genes and environment conditions both. Hence, selection of the engineered bacterial strain, with fast growth and high metabolic versatility, having high bioremediation potential without environmental risk will facilitate as a critical step in attaining a secure and sustainable environment. Examples of engineered bioremediation techniques are bioventing, biosparging, bioslurping, biostimulation, and bioaugmentation.

Bioventing

Bioventing is a mode of engineered in situ bioremediation that accelerates the natural biodegradation of some aerobically degradable pollutants, i.e., non-chlorinated volatile organic compounds (VOCs) and semivolatile organic compounds (SVOCs) such as petroleum hydrocarbons), that are situated in the vadose (unsaturated) zone, by delivering air/oxygen to prevailing indigenous aerobic microbes (USEPA 2006). Bioventing uses minimal flow rates of air and provides a limited amount of oxygen required for the biodegradation though minimizing volatilization and discharge of pollutants to the ecosystem. Generally, a bioventing system supplies air/oxygen from the environment into the anaerobic and permeable polluted soil above the water level through instilment wells positioned on the ground where the pollutants persists (Fig. 6.2a). This technique is found to be more effective if the contaminated sites have high temperature and the water level is lower from the surface.

Biosparging

Biosparging is an in situ remediation method which implies the instilment of air under pressure below the water level in order to elevate the groundwater oxygen concentrations and enhance the disintegration rate of organic contaminants in the saturated zone by indigenous microbes (Fig. 6.2b). This procedure enhances the biological activity in contaminated soil and to endorse aerobic microbial degradation by augmenting the oxygen supply via sparging air or oxygen into the soil.

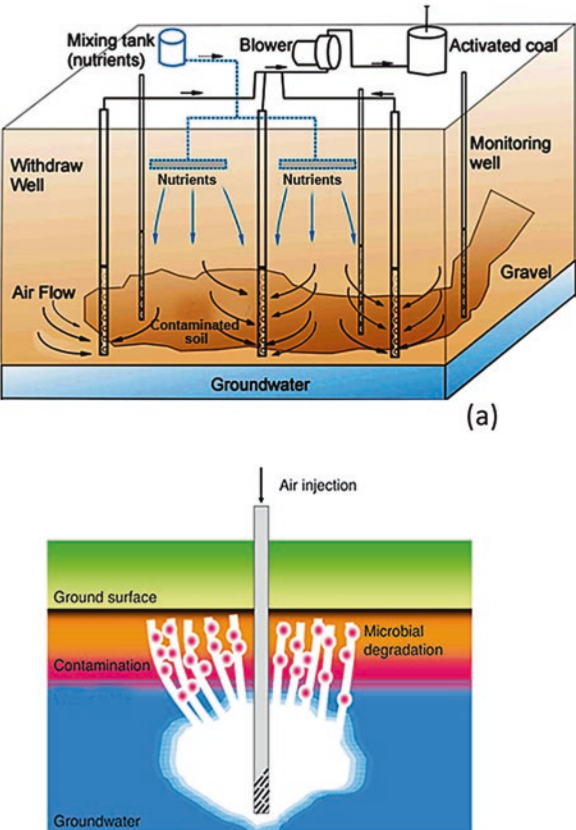

Fig. 6.2 Schematic diagram's illustrating treatment strategies involve in bioremediation processes: (**a**) bioventing and (**b**) biosparging (Antizar-Ladislao 2010)

Biosparging increases the mixing within the saturated zone and thus upsurges the contact between groundwater and soil (USEPA 2006). It is commonly exercised on the sites contaminated with lighter petroleum products like gasoline, mid-weight petroleum products (e.g., jet fuel, diesel fuel) which are readily volatile and needed to be eliminated more promptly by air sparging.

Bioslurping

Bioslurping, a multiphase extraction, is an effective in situ remediation method that merges vacuum augmented free product recovery by bioventing of subsurface soil to simultaneously remediate soil, sediment, and groundwater which is polluted with PAHs (Fig. 6.3). Bioventing promotes aerobic disintegration of pollutants persisting in the contaminated soil, while vacuum-enhanced recovery uses the negative pressure to form a partial vacuum which removes free product and water from the subsurface. Bioslurping is usually used on the petroleum spill sites and has found to be

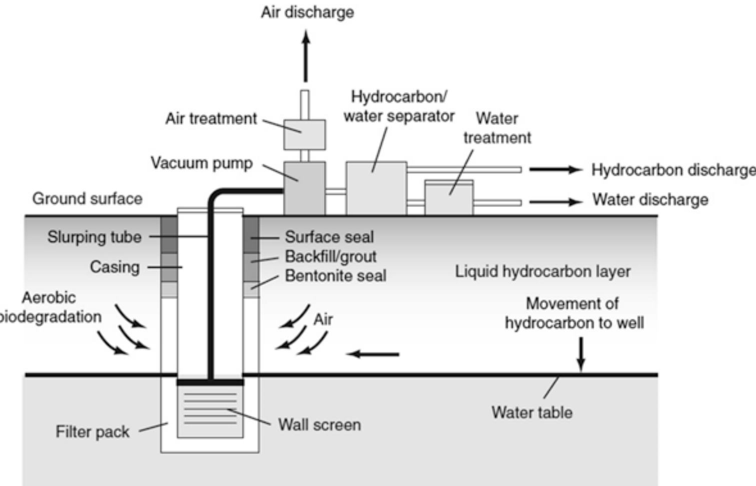

Fig. 6.3 A schematic view of bioslurping technology (USEPA 2006)

most operative on fine-to-medium-textured soils or fractured rock within areas having low water level (USEPA 2006).

Biostimulation

Biostimulation is often described as the supplementation of nutrients, electron donors, or electron acceptors to the contaminated site with the intention to stimulate the growth and metabolic activity of indigenous chemical-disintegrating microbial community or to advance co-metabolism (Tyagi et al. 2011). The notion of biostimulation is to hasten the naturally occurring biodegradation process under ideal physicochemical conditions, like appropriate pH, temperature, oxygen/air, moisture and water content, etc., nutrients, and the addition and/or presence of probable microbes. This method has been employed for the remediating wide variety of xenobiotics. Biostimulation comes under the "enhanced bioremediation" methods accompanied by "bioaugmentation" which involves the inoculation of specific indigenous or nonindigenous microbes intending to accelerate the biodegradation rate of aimed pollutants.

Bioaugmentation

Bioaugmentation is one of the in situ bioremediation strategies, which aims to improve the biodegradative abilities of polluted sites by inoculating indigenous or allochthonous wide form or genetically altered bacterial strains or microbial consortia having preferred catabolic abilities to disintegrate recalcitrant compounds in such habitat (El Fantroussi and Agathos 2005). It is assumed that bioaugmentation is always executed in combination with biostimulation. The chemical structures of numerous toxic molecules can be so complex that consortium of assorted microbes will be needed for their biodegradation, or all of the microbes essential may not be

simultaneously prevailing in the contaminated sites. In many cases, recalcitrant molecules may be novel, and thus, microbes may not have yet amended to exploit them as a substrate. Bioaugmentation can conquer these challenges, as one of its main benefits is that treatment can be amended to a specific pollutant which is ascendant in the environment.

6.2.2 Ex Situ Bioremediation

Ex situ bioremediation method involves the digging of the contaminated media like soil, sediment, or sludge pushing of groundwater to facilitate microbial degradation of pollutants. Ex situ bioremediation can take place in two ways: (1) slurry-phase bioremediation and (2) solid-phase bioremediation.

6.2.2.1 Slurry-Phase Bioremediation

Slurry-phase bioremediation is a biological procedure where the contaminated soil, sludge, or sediment is blended with H_2O and other chemicals within a bioreactor, a container in which three phases are created like liquid, solid, and gas (three-phase) and blending conditions are maintained to elevate the biodegradation rate of water-soluble and soil-connected pollutants present in the water slurry of the contaminated soil, sludge, or sediment and biomass of indigenous microbes. It is blended so as to keep the microbes in association with the hazardous contaminants present in the substrates. Then oxygen and nutrients are supplemented to the reactor to establish the ideal environmental conditions for microbes to degrade the specific pollutants. Once the process gets completed, the H_2O is withdrawn from the soil, and the soil is assessed and replenished in the environment (USEPA 2006). This method is relatively rapid as compared to other bioremediation methods. In slurry-phase bioremediation, the rates of pollutants disintegration are effective in a bioreactor treatment system than in solid-phase systems in situ since the enclosed environment is more manageable, controllable, and predictable.

6.2.2.2 Solid-Phase Bioremediation

Solid-phase bioremediation is a method which treats the contaminated soil within an aboveground treatment area. Conditions within the treatment areas are monitored in order to ensure optimum treatment is taking place. This kind of treatment is easy to uphold, but it needs a lot of space, and the course of decontamination takes longer as compared to slurry-phase bioremediation. The theory of solid-phase bioremediation is based on the mechanical breakdown of polluted soil by abrasion and by an intensive blend of the components in an enclosed vessel. This confirms that the nutrients, microbes, pollutants, O_2, and H_2O are in permanent contact. Solid-phase soil treatments include soil biopiles, land farming, and composting practices for detoxification and disintegrating the hazardous toxic contaminants.

Land Farming

Land farming, also known as land application/land treatment, is an ex situ treatment method in which contaminated soil, sediment, or sludge is digged and dispersed on a prepared bed and cyclically turned over (tilled) for aerating the mixture till contaminants gets degraded via stimulated aerobic microbial activities in the soils due to aeration and/or the supplementation of moisture, minerals, and nutrients. This practice is restricted for the treatment of superficial 10–35 cm of soil (USEPA 2006). The land farming method has been verified to be efficient in lowering the concentrations of all the components of petroleum products usually found on underground storage tank sites.

Composting

Composting is a biological disintegration process in which organic wastes are transformed into humus-like matters by microbes, which is stable organic end product (compost). In composting, the contaminated soil is dug out and blended with a bulking agent and organic materials (such as animal wastes, wood chips, vegetative wastes, etc.). The existence of these organic constituents aids the proliferation of a rich microbial community which changed the organic matter into compost via their enzymatic activity. Usually, composting is the anaerobic, thermophilic procedure of microbiological disintegration of polluting agents (organic wastes) into stable end product (usually compost) which can be disposed safely into the environment. Under normal environmental circumstances, earthworm; soil insects, i.e., mites, sow bug, ants, springtails, and beetles; and nematodes start the degradation of organic material into minute particles, thus intensifying their bioavailability for the microbial community, whereas, under regulated environmental conditions, composting machinists disintegrate the large waste entities via chopping or grinding. A huge number of microbes are involved in the disintegration of organic contaminants that are readily available in the wastes. Soil microbes like bacteria, fungi, actinomycetes, and protozoa are acquainted when the wastes are blended with soil or inoculated with finished compost. The composting process is executed by three classes of microbes: (1) psychrophiles, (2) mesophiles, and (3) thermophiles. For profitable composting, microbes require nutrients, moisture, temperature, and oxygen. During composting, microbes degrade the organic compounds to acquire energy for carrying out the metabolic activities and obtain nutrient (N, P, and K) for their endurance. Among many elements which are essential for microbial disintegration, C and N are the most important. The model C:N ratio for composting is thought to be about 30:1. As composting continue, the C: N ratio gradually declines from approximately 30:1 to 10–15:1 to reach the finished product. Usually, composting commences from mesophilic temperatures and advances toward the thermophilic range. In most of the cases, composting is accomplished with the help of indigenous microbes. The wastes are formed to less complex materials which are lower in mass. The aeration, moisture, and temperature are carefully monitored to achieve higher degradative efficiency.

Biopiling

Biopiling, also called biocells, biomounds, or bioheaps, is an ex situ bioremediation method in which burrowed soil, sludge, or sediments are blended with soil amendments, placed on a treatment area, and remediated using forced aeration. This method encompasses the stacking of contaminated soil, sludge, or dried sediments into piles and accelerating the biodegradation activity of aerobic microbial community by forming ideal proliferating conditions within the pile (Germaine et al. 2012). Biopiles are generally 2–3 meters in height, and contaminated soil, sludge, or sediment is conventionally laid on top of the treated soil. This methodology is extensively employed for the remediation of wide range of diesel, crude, and lubrication oil contaminated soils, sludge, or sediments (Das and Dash 2014). The primary procedure of toxic waste eradication in biopiles is by stimulating the metabolic activities of pollutants degrading microbes by the supplementation of nutrients (carbon and nitrogen) and diffusion of oxygen within the soil. Soil microbial activity can also be augmented in biopile soil through direct addition of pollutants degrading microbes.

6.3 Mechanism of Bioremediation

Bioremediation is a biodegradation procedure in which sites polluted with hazardous pollutants are cleaned up with the help bacterial biogeochemical processes, by utilizing the ability of microbes in reducing the concentration and/or toxicity of a large variety of pollutants (Kumar et al. 2017). In common words, biodegradation means the mineralization of organic components into soluble inorganic compounds or conversion of organic components to other soluble organic compounds. In biodegradation procedure, a wide variety of microbial enzymes that take part in the transformation of both natural and artificial organic contaminants into intermediate mixtures which may be similar or less hazardous to that of their parental compounds. A large number of fungi, bacteria, and actinomycetes genera owing to their vast catabolic potential and biodiversity have been developed to biodegrade the toxic contaminants. These diverse catabolic activities in microbes are due to the presence of diverse enzymes and catabolic genes. In addition, microbes possess other adaptation tactics like they can use the efflux pumps to reduce the concentration of toxic compounds within the cell, produce biosurfactants, and amend the cell membrane to sustain the necessary biological functions. All these mechanisms and metabolic abilities make microbes as a latent cleanup tool for the bioremediation of waste contaminated sites.

Microbes interact physically and chemically with contaminants heads toward the structural alterations or complete disintegration of the target pollutants. The disintegration of organic pollutants is centered to two processes: growth and metabolism. During the bioremediation, microbes utilize the organic contaminants for their proliferation. In addition, other major nutrients, such as N and P, and minor nutrients like sulfur (S) and trace elements are also necessary for their proliferation. Thus, it provides the electrons, which these microbes can use to gain energy. This procedure

results in complete disintegration of organic compounds. In addition, microbes obtain energy by catalyzing energy-generating chemical reactions which involve dissociation of chemical bonds and transferring electrons away from the pollutant. These forms of chemical reaction are known as an oxidation-reduction reaction: the organic contaminant gets oxidized via losing electrons (electron donor), while the chemical which gains the electrons gets reduced (electron acceptor). The energy acquired by these electron transfers is then "spent," with some electrons and carbon from the pollutant, to generate more new cells. These two constituents (electron donor and acceptor) are important for cell proliferation and the primary substrates.

Microbes can utilize an amalgam of electron donors and electron acceptors reactions to propel their metabolism. In addition, they have also refurbished myriad of other strategies which enable them to cleanse the environment. The metabolism means of microbes have generally classified them into two types (1) aerobic and (2) anaerobic. Aerobic processes of terminating organic molecules take place in the presence of molecular oxygen, whereas microbes use molecular oxygen as the terminal electron acceptor. This type of metabolism is called as aerobic respiration. In aerobic respiration, microbes spend oxygen to oxidize the carbon of contaminant to CO_2, whereas the remaining carbons are used to synthesize new biomass. Anaerobic reactions take place during the absence of molecular O_2, and the reactions are subcategorized into anaerobic respiration, fermentation, and methane fermentation. In anaerobic respiration, microbes consume oxidized inorganic or organic molecules except O_2 as the terminal electron acceptor. Disintegration of organic contaminants by microbes takes place either during the presence of oxygen for respiration or under anaerobic (anoxic) conditions with the help of denitrification, methanogenesis, and by sulfidogenesis (Fig. 6.4). In the nature, the fast and complete disintegration of the widely used contaminants is achieved under aerobic condition. The primary enzymatic reactions which take place during aerobic biodegradation are oxidations, catalyzed through oxygenases and peroxidases, and use oxygen to incorporate into the substrate. Biodegradative organisms require oxygen at two metabolic stages (1) during initial attack on the substrate and (2) on completion of

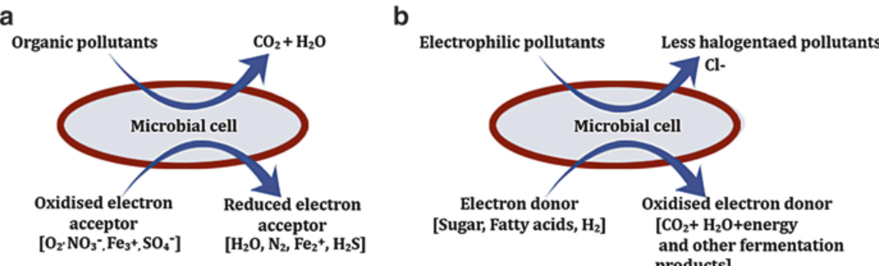

Fig. 6.4 Schematic view of the biodegradation of organic pollutants. (**a**) In oxidative biodegradation, pollutants are oxidized by external electron acceptors such as oxygen or sulfate. (**b**) In reductive biodegradation, electrophilic halogen or nitro groups on the pollutant are reduced by microbes consuming sugars, fatty acids, or hydrogen. The halo or nitro group on the pollutant serves as the external electron acceptor (Rockne and Reddy 2003)

the respiratory chain. Under regulated anaerobic conditions, soluble carbon molecules are disintegrated stepwise, into CO_2, methane (CH_4), ammonia (NH_4+), and hydrogen sulfide (H_2S) with the help of fermentative and acetogenic microbes, methanogens, or sulfate reducers. Utmost information related to biodegradation of organic contaminants involves oxidative degradation. In aerobic process, O_2 availability increases the proliferation rate and yield of aerobic microbes. Aerobes produce monooxygenases and dioxygenases, which are helpful in the oxidation of hydrocarbons. The availability of oxygen, also, suppresses the anaerobic processes, like the disintegration of halogenated contaminants, by the inhibition of reductive dehalogenation. During hydrocarbon degradation, O_2 gets rapidly exhausted at heavily contaminated sites and develops anaerobic conditions. Anaerobic activity is prevalent and has been reported under nitrate-, iron-, manganese-, and sulfate-reducing conditions, also under methanogenic conditions.

In the natural conditions, it often seen that these degradation processes are escorted by transformations of other molecules, other xenobiotics. This phenomenon is also explained by using various terms, like co-metabolism, co-oxidation, gratuitous metabolism, and free or accidental metabolism. Co-metabolism means metabolism of an organic molecule with no nutritional gain in the presence of a growth substrate, which is consumed as the primary carbon as well as an energy source. It is a regular phenomenon of microbial activities. Bacteria secrete metabolic enzymes which degrade the complex organic material encircling them for easier digestion. These enzymes are generally nonspecific and, thus, can function on several different types of substrate materials, involving those which are not useful for bacteria itself for energy. Enzymes like methane monooxygenase and ammonia monooxygenase are examples enzymes which can oxidize a broad range of substrates (Hazen 2009). Co-metabolic treatment possibly can measure contaminant of trace levels, till the substrate that is available for bacterial growth is sustained at adequate concentrations as these bacteria do not depend on this contaminant for energy. Co-metabolism was endorsed for the treatment of TCE, but now it is rarely used as the intermediate epoxide synthesized which further obstructs biological activity. The TCE oxidation by-products like TCE epoxide result in the inactivation of the oxygenase activity by impairing the enzymes. Inhibition as well as inactivation can be overcome by adding the natural substrates. Co-metabolism may ascertain for treating other problematic pollutants like *N*-nitrosodimethylamine (NDMA) and 1,4-dioxane. Figure 6.5 illustrates the co-metabolic degradation of trichloroethylene.

6.4 Plant-Assisted Bioremediation (Phytoremediation)

Plant-assisted bioremediation, or phytoremediation, is an in situ, eco-friendly, solar-powered evolving methodology which uses plants and associated rhizospheric and/or endophytic microbes to disintegrate, remove, sequester, transform, metabolize, assimilate, or detoxify pollutants present in the soil, sludge, sediments, groundwater, and surface water (Germaine et al. 2012; Segura and Ramos 2013). These plants

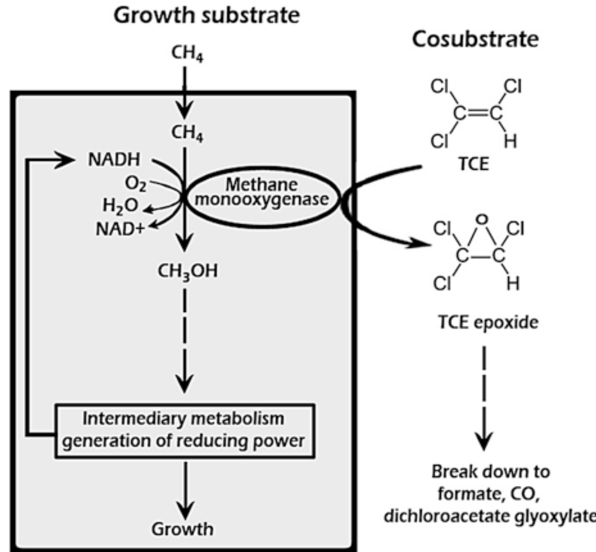

Fig. 6.5 Co-metabolic degradation of trichloroethylene by microbial cell

can consequently be harvested, processed, or disposed of safely. Plants have the ability to uptake pollutants retaining in the environment via the root system, which, by providing a larger surface area, facilitate mobilization, clean up, or detoxification of contaminants within plants through various mechanisms, i.e., elimination, containment, degradation, etc. Such plant properties have been used for effective elimination of wastes, including metals, phenolic compounds, azo dyes, and colorants, and various other organic and inorganic contaminants. The microbial population allied with plant and plant-microbe interactions formed among them have a critical role in the maintaining physiology as well as health of the plant, exerted to inhibit phytopathogens by releasing the growth-promoting compounds, enhance the nutrient availability, promote detoxification (e.g., degradation, sequestration, and volatilization of pollutants), and improve the stress tolerance by introduction of systematic acquired host resistance. Plants provide leaves, stems, and roots, as habitats for a broad range of microbes which readily degrade the toxic pollutants and elevate the treatment process. Plants use different mechanisms such as phytoextraction, phytostabilization, phytodegradation, phytovolatilization, rhizodegradation, and rhizofiltration to uptake different organic and inorganic pollutants, which make the basis of phytoremediation technology (Fig. 6.6) (Tangahu et al. 2011).

6.4.1 Phytoextraction

Phytoextraction (also known as phytoabsorption, phytosequestration, or phytoaccumulation) is a cost-effective technique in which plant roots uptake metal contaminants from soil, water, or sediment and then transport it from roots to shoot and leaves of the plants (McGrath and Zhao 2003; Salt et al. 1998). Several natural

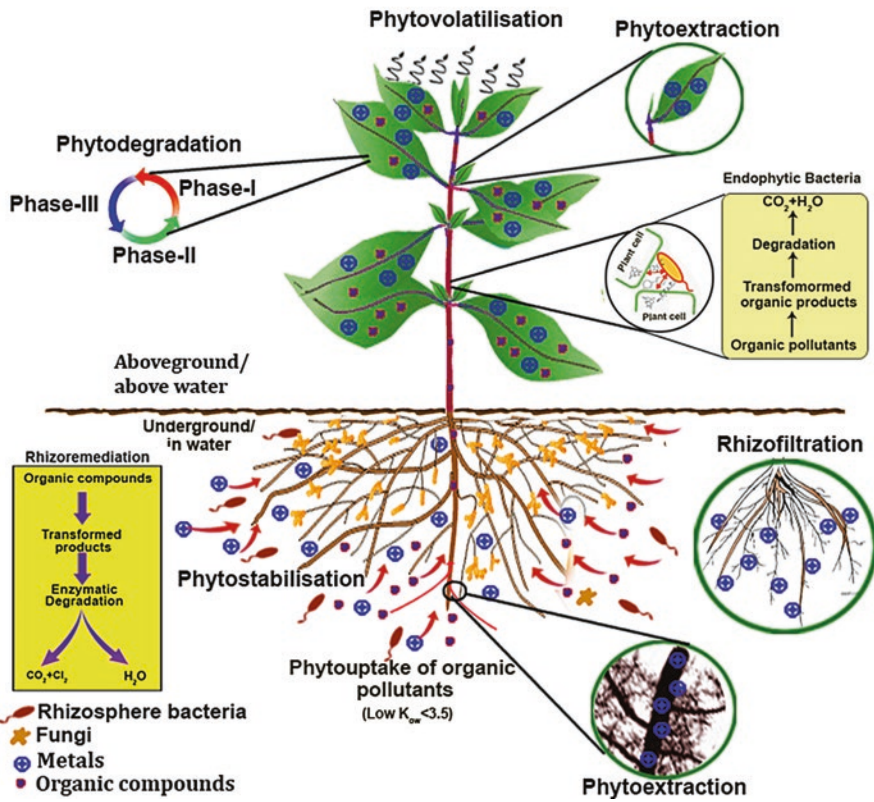

Fig. 6.6 Schematic presentation of various phytoremediation strategies involved in remediation of organic and inorganic pollutants from contaminated environment

plants, called hyperaccumulators, accumulate and tolerate a huge amount of metals/metalloids in their shoot without any visible toxic symptoms compared to other plants (Garbiscu and Alkorta 2001). Hyperaccumulator plants employ varied metabolic processes for the uptake and mobilization of metal ions from polluted soil, sludge, or sediment, metal is translocated from root to shoots, sequestration of metal ions within tissues and cells, and alteration of hoarded metal ions into less toxic and/or harmful forms (Kumar et al. 1995). After accumulation of metals, plant biomass is harvested and either incinerated or composted to recycle the metals. An ideal hyperaccumulator plant would have high biomass and rapid growth, high capability to accumulate heavy metals/metalloids in their shoots, and BCF (bioconcentration factor) and TF (translocation factor) value greater than one (>1) (McGrath and Zhao 2003). BCF and TF are essential parameters in heavy metal uptake studies in plants (Yoon et al. 2006). BCF endows an index about the ability of the plants to hoard a specific metal in regard to its concentration in the soil, sludge, or sediment, whereas TF represents the ratio of metal concentration present in the shoot in comparison to the root (Yoon et al. 2006; Gupta and Sinha 2007). A plant species with both BCF

and TF values >1 is potential for phytoextraction of particular metals from contaminate sites (Yoon et al. 2006). Besides, plant with BCF >1 and TF <1 (less than one) has the potential for phytostabilization (Fitz and Wenzel 2002).

6.4.2 Phytostabilization

Phytostabilization in an in situ technology uses certain plant species to immobilize heavy metals in soil, sediment, or sludge through accumulation, precipitation within the rhizosphere, and adsorption onto roots or physical stabilization of soil, sediment, or sludge. This method reduces metal movement and leaching into groundwater and moreover reduces the chances of metal ions to enter our food chain (Yoon et al. 2006). In addition, this method reduces the motion of pollutants and avoids their migration into groundwater or air. One advantage of phytostabilization technology is that the disposal of the metal-containing plant material is not required. This can restore the polluted sites where natural vegetation doesn't sustain because of the high metal concentrations (Tordoff et al. 2004).

6.4.3 Phytodegradation

Phytodegradation (also called as phytotransformation) refers to uptake and degradation of organic pollutants within the metabolic competences of plant and their allied microbes or the disintegration of organic contaminants in the soil, sludge, surface water, or groundwater with the help of enzymes like dehalogenase, peroxidase, nitroreductase, laccase, and nitrilase (McGrath and Zhao 2003). However, a metabolic progression known as ex planta ensues when organic pollutants gets absorbed by plants and degraded into smaller entities via plant enzymes. The produced monomer compounds are incorporated into plant tissues to aid plant growth (McGrath and Zhao 2003). From this point of view, green plants can be regarded as "green liver" for the biosphere. This technology can be used to degrade organic pollutants, trichloroethylene (TCE), polychlorinated biphenyls (PCBs), 2,4,6-trinitrotoluene (TNT), polycyclic aromatic hydrocarbons (PAHs), BTEX, naphthalene, phenol herbicides, and pesticides. Phytodegradation is most appropriate for moderately hydrophobic organic compounds (octanol-water partition coefficients, log K_{ow} = 0.5 ~ 3.5.). The octanol-water partition coefficient (K_{ow}) is an important physicochemical characteristic widely used to describe hydrophobic/hydrophilic properties of chemical compounds. It is related to the transfer free energy of a compound from water to octanol. In the plant, the K_{ow} was represented as determining factor for root access and translocation of organic molecules. Organic contaminants having value of log K_{ow} < 1 are regarded to be highly water-soluble, and plant roots do not amass them at the flow of surpassing passive influx toward the transpirational pull, whereas organic contaminants having value of log K_{ow} > 3.5 illustrate high sorption through plant roots and, however, show slow or no translocation from root to shoot. Hence, plants eagerly pick up organic molecules having value of log K_{ow} between

0.5 and 3.5 (Trapp et al. 2000), once these pollutants are absorbed by plants, it gets metabolized by these pollutants even though few of them or their metabolites are toxic (Doucette et al. 1998).

6.4.4 Phytovolatilization

Phytovolatilization is a removal technique in which metabolic capabilities of plants and their rhizospheric microbes are used to alter the toxic contaminants into volatile forms (less toxic/nontoxic) which are then discharged into the atmosphere. In phytovolatilization, metals from the soil are uptaken by the roots of plant and liberated into the environment through the transpiration process. This process usually works when nature of metals is volatile (Hg and Se). The main advantage of phytovolatilization is that it can remove the contaminants from the site, without harvesting the plant and disposal, in comparison to other cases. Phytovolatilization is one of the most debated phytoremediation methods as Hg and Se are toxic, and there is suspicion whether volatilization of these toxic metals is safe for discharging into the atmosphere.

6.4.5 Rhizodegradation

Rhizodegradation (also called rhizoremediation, phytostimulation, microbe-assisted phytoremediation) is a process in which plant supplied substrates to stimulate the growth of microbial communities in rhizosphere the breakdown of organic pollutants in soil (Vishnoi and Shrivastava 2008). Plant rhizosphere provides a unique environment for microorganisms that are capable of breaking down hazardous pollutants into the nontoxic and harmless product through their metabolic activity. In this process of rhizodegradation, plant released nutrient in the form of root exudates. Due to the presence of these exudates, the microbial communities and their activity in the rhizosphere can increase and can result in increased degradation of organic pollutants in the soil. Rhizospheric microbes, in turn, enhance the plant growth development by nitrogen fixation, mobilization of nutrients, production of plant growth regulators, providing protection against plant pathogens, decreasing plant stress hormone levels, and degradation of pollutants before they negatively impact the plant growth. In various cases, rhizosphere microbes are found to be major contributors for pollutant degradation process. The microbial augmented rhizoremediation process is significantly slower in comparison to ex situ actions because of environmental restrictors present at sites like competition among weed, restricted plant growth in the polluted environment, the manifestation of plant pathogens and other abiotic/biotic stressors. Moreover, rhizoremediation is operative only in root zone and is inappropriate for the treatment of deeper subsurface layers.

6.4.6 Rhizofiltration

Rhizofiltration is an in situ or ex situ root zone technology in which plant roots to absorb, precipitate, and accumulate pollutants, mainly toxic metals such as Pb, Cd, Cu, Ni, and Cr, from polluted water and effluent and aqueous waste streams (USEPA 2000a, b). An ideal plant for rhizofiltration should have fast-growing root system with the ability to eliminate metals from solution over an extended period of time. However, rhizofiltration worked with several physical and biochemical processes such as adsorption, precipitation, rhizodegradation, and bioaccumulation. Rhizofiltration of metals involves the absorption into or precipitation onto plant roots of the metals present in complex industrial wastewater. In addition, other slower biological processes such as intracellular uptake, translocation to the shoot and deposition in vacuoles or precipitation of the metal from wastewater by plant exudates also occur in rhizofiltration. Based upon the nature of pollutants present in wastewater, rhizofiltration process may take place with phytoextraction, phytostabilization, or phytovolatilization process.

6.5 Factors Affecting Bioremediation

From the environmental point of view, bioremediation depends on the various interactions between three factors: pollutant, organisms, nutrient, and environment. The interactions of these factors affect biodegradability, bioavailability, and physiological requirements, which are important in assessing the feasibility of bioremediation. Most important parameters that affect biodegradation processes are:

1. Bioavailability of pollutants—the pollutant can interact with its surrounding environment to change its bioavailability or availability to organisms that are capable of degrading it. Bioavailability is different between species and organisms, so it can be defined in terms of specific organism as the extent to which pollutant is free to move into or on to organism. The in situ microbial degradation of organic pollutant is a function of bioavailability of contaminant and catabolic activity of microbes.
2. Nutrients—the nutrient availability affects the microbial transformation and detoxification process of contaminants included direct inhibition of proliferation process and enzymatic activities of pollutant degrading organisms. In addition, microbes need minerals like nitrogen, phosphate, and potassium (N, P, and K) as nutrients for cellular metabolism and efficacious proliferation at the polluted sites. At industrial waste polluted sites, level of organic carbon is often high because of the natural contaminants, promptly available nutrients get rapidly degraded during microbial development. The nutritional requisite C:N (carbon-to-nitrogen ratio) should be 10:1, and C:P (carbon-to-phosphorous ratio) should be 30:1 during degradation (Atagana et al. 2003).

3. Environmental conditions like pH, temperature, salinity, pressure, oxygen, water activity, and moisture availability vary from site to site and impel the process of bioremediation via inhibition of the growth of contaminant-degrading microbes. Under ideal environmental conditions, the microbes divide and proliferate in the contaminated environment and metabolized more pollutants. If environmental conditions are unfavorable, microbes grow too slowly or die, and contaminants are not cleaned up. pH is one of the most important environmental factors, and a pH of 6.5–8.5 is optimal for biodegradation of pollutants in most terrestrial and aquatic ecosystem (Jourtey et al. 2013). Furthermore, oxygen also plays a critical role in the biodegradative process. The solubility of organic molecules specifically PAHs surges by the temperature, which, conclusively, increases its bioavailability to microbes. In addition, O_2 solubility declines with increase in temperature, which reduces the development and metabolic activities of aerobic microbes. In addition, moisture is also crucial for all processes that take place inside the cell to the transportation of waste products, foods, water, and nutrients, inside and outside the microbial cell. It influences the rate of contaminant metabolized by microbes as it influence the amount and type of soluble constituents that are available with the pH and osmotic pressure as that of the terrestrial and aquatic systems (Vidali 2001).
4. The capability of the soil's microbial population to degrade contaminants in polluted site depends on the number of microbes and its catabolic potency. Meanwhile, soil microflora contains numbers of algae, fungi, protozoa, and actinomycetes, which performs different metabolic activities for assailing hydrocarbons. The soil microbes usually range from 10^4 to 10^7 CFU; for effective biodegradation, the number should not get reduced from 10^3 per gram of soil. Microbes having numbers $<10^3$ CFU per gram of soil signal the presence of toxic contaminants (Margesin et al. 2003; Pawar 2012). The activity of soil microbes can be regulated by nutritional as well as environmental factors. For effective biodegradation, it is necessary that the microbes should improve catabolic activities by genetic alteration, introduction of specific enzymes, and selective supplementation for organisms so that they transform the target molecules (Pawar 2012).

6.6 Advantages and Disadvantages (Limitations) of Bioremediation Technology

Bioremediation is the exploitation of living organisms (primarily microbes) to disintegrate the hazardous environmental contaminants into less toxic state. The final products of bioremediation such as water, carbon dioxide, and microbial biomass are nontoxic to the environment and living organisms. There are several key advantages and disadvantages (limitations) to using bioremediation technology which are summarized in Table 6.1.

Table 6.1 Advantages and disadvantages (limitations) of bioremediation

S. No.	Advantages	Disadvantages
1.	Bioremediation takes advantage of the natural ability of microorganisms to extract chemicals from water, soil, and sediment using energy from sunlight	The process of bioremediation is slow. Treatment time is typically longer than that of other remediation technologies
2.	It is a cost-effective technique compared to other physicochemical treatment methods	It does not remove all quantities of contaminants from the polluted site
3.	Less energy is required as compared to other technologies	Bioremediation is not useful for treatment of inorganic contaminants or every organic compound
4.	Often little to no residual treatment is required, whereas in physical/chemical treatment, the contaminants are often just separated	For in situ bioremediation site must have soil with high permeability
5	Typically lower cost to implement	Performance evaluations are difficult because there is not a defined level of a "clean" site, and therefore performance criteria regulations are uncertain. Difficult to determine whether contaminants have been destroyed
6.	Bioremediation can be done on site and is often less expensive, and site disruption is minimal as compared to conventional remedial methods	In some cases, some compounds may be broken down through microbial metabolism into more toxic metabolites/by-products (e.g., TCE to vinyl chloride) or PAHs to less degradable PAHs (carcinogens). These may be mobilized to groundwater if not controlled
7.	Enhanced regulatory and public acceptance	If an ex situ process is used, controlling of volatile organic compounds may be difficult
8.	Soil stabilization and reduced water leaching and transport of organic compounds in the soil	Some chemicals are not amenable to biodegradation, for instance, heavy metals, radionuclides, and some chlorinated compounds

6.7 Future Perspectives and Concluding Remarks

Bioremediation is an adaptable, eco-friendly treatment approach and a fast-growing field of environmental restoration. It uses the microbial capability to disintegrate and/or detoxify environmental pollutants into less toxic forms. The presence of potent microbes for a particular bioremediation requires nutrient in conjunction with suitable growth conditions, established by in vitro and field experiments; this kind of information allows us to manipulate the environmental conditions which can constrain the biodegradation of organic contaminants for in situ treatments. Additionally, a profound insight of microbial degradation capabilities, along with their metabolic processes, adaptation, and resistance mechanisms, will reveal the variation of suitable "microbial formula" which can be amended according the

specific polluted site. It is important to highlight that numerous field experiments have not been regulated properly, designed correctly, or analyzed properly, which has led to ambiguity. Thus, imminent field studies should permit stern endeavors by adapting scientifically proven approaches and attain the possible high-quality data. The understanding of microbial diversity of the contaminated site is important to procure an improved insight about potent degraders as well as let us comprehend their biochemistry and genetics which will facilitate in evolving suitable bioremediation approaches. The inadequate information about the microbes along with their function in the environment could influence the suitability of their uses. Exploitation of bacteria for bioremediation of ROPs needs an awareness about all physical and biochemical attributes which are implied during biochemical and transformations. Imminent investigation should focus on the interpretation of the particular mechanisms via which the organic pollutants are metabolized by the indigenous bacterial communities. The mechanisms involved in the movement of organic pollutants inside the bacterial cells, disintegration pathways, and introduction and regulation of degrading enzymes should be researched. The future exploration should also focus on the enzyme systems and the location of genes implied during the disintegration of the organic pollutants. The approach of biomolecular engineering should be developed in order to enhance the degradation abilities of the microbes or enzymes involved in bioremediation process. The genetically altered bacteria with improved abilities to degrade diverse types of pesticides in field conditions can be generated by bimolecular engineering. Furthermost, the advances in bioremediation have been attained due to the support of different scientific areas of biochemistry, microbiology, analytical chemistry, molecular biology, environmental engineering, and along with others. These different fields, each encompassing individual approach, have vigorously contributed toward the expansion of bioremediation process in recent years.

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7 Myxobacteria: Unraveling the Potential of a Unique Microbiome Niche

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Abstract

Natural products obtained from microorganisms have been playing an imperative role in drug discovery for decades. Hence, rightfully, microorganisms are considered as the richest source of biochemical remedies. In this review, we represent an unexplored family of bacteria considered to be prolific producers of diverse metabolites. Myxobacteria are gram-negative bacteria which have been reported to produce large families of secondary metabolites with prominent antimicrobial, antifungal, and antitumor activities. Klaus Gerth, Norbert Bedorf, Herbert Irschik, and Hans Reichenbach observed the antifungal activity of *Sorangium cellulosum* against *Mucor hiemalis*. In 2006, Hans Reichenbach and his team obtained a novel macrolide cruentaren A from *Byssovorax cruenta* (myxobacteria). Cruentaren A showed inhibitory activity against yeast and filamentous fungi. It also showed selective inhibitory activity against mitochondrial F-type ATPase. Cruentaren A has been found to be cytotoxic against various human cancer cell lines.

In 2007, Reichenbach and his colleagues named an antibiotic produced by *Sorangium cellulosum* strain Soce895 as thuggacin. This antibiotic acts on the respiration of some bacteria. Other antibiotics from myxobacteria, myxovirescin, and megovalicin show broad-spectrum bactericidal activity. The College of Food and Bioengineering, Henan University of Science and Technology, Luoyang, China, evaluated the antitumor property of epothilone, which has shown promise for breast cancer treatment. The study determined high potential and versatile antimicrobial and antitumor secondary metabolites of myxobacteria. In yet another study, Ratjadone A, that exhibited strong antiviral activity against HIV, was obtained from *Sorangium cellulosum* strain. This compound shows antiviral activity in vitro but has low selectivity. Further search on the derivatives

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of this compound might help in the future. This is rationale enough to pre-empt that every strain of myxobacteria might be endowed to produce secondary metabolites with novel mechanisms of action which are rarely produced by other microbes. The available data establishes the impact of myxobacterial studies in search for novel metabolites as a front runner in microbiological research and worthy enough to be a thrust area of research in pharmacology.

Keywords
Gram-negative · Metabolites · Myxobacteria · Anticancer reagents · *Sorangium cellulosum*

7.1 Introduction

Microorganisms are extremely diverse group of organisms which cover about 60% of the Earth's biomass. Myxobacteria are one of the relatively unexplored species of microbes. This is one of nature's "endowed" and extensively dispersed microorganisms in the ecosystem. They are distinctively slow-growing, gram-negative, rod-shaped, aerobic, chemoorganotrophic bacteria, famous for their unique developmental life cycle and development of multicellular fruiting bodies, which is an important character for myxobacteria classification. The optimum temperature for growth of these bacteria is approximately 26–34 °C, but some strains can also grow above and below the optimal temperature range. The *Myxococcus* strain of myxobacteria is found to grow at temperature 6 °C and near neutral pH. Under harsh conditions, cluster of vegetative cells form multicellular fruiting bodies that contain dormant myxospores.

Myxobacteria are distinguished from other bacteria courtesy of their complex developmental biology and remarkable social behavior in swarms. In comparison with other gram-negative bacteria, they consist of lipopolysaccharide component in their outer membrane, but they differ in the presence of menaquinones in their respiratory chains. Based on morphological and physiological parameter, myxobacteria are divided into two groups as the *Cystobacterineae* and *Sorangineae* within the order *Myxobacteriales* (*Myxococcales*). This classification was based on the ability of the bacterial cells to bind with the diazo dyes as Congo red, considered as one of the reliable classification tests for myxobacteria. The difference in Congo red dye binding is due to the variability into polysaccharide structure.

They act as microbial predators that control the population of soil microbes. Predation properties of myxobacteria involve secretory diffusible factors and direct cell to cell contact. Vegetative myxobacteria utilize their prey by secreting antibiotics and hydrolytic enzymes. These microorganisms uptake their nutrients by extracellular digestion of their prey. This observation gave new term that myxobacteria feed as microbial "wolf packs," i.e., as the cell density increases antibiotic and extracellular hydrolytic enzymes concentration also increases, that allow more efficient prey killing**.** These unusual bacteria are found to be abundant producers of

bioactive compounds with immense value that include antibacterial, antifungal, antiviral, and antitumor compounds with novel mechanisms of action which are rarely produced by other microbes**.** *Myxococcus* group has also obtained consideration and recognition for production of novel drugs and chemotherapeutic agents that show remarkable modes of action. Unexpectedly, myxobacteria were also reported for their prospects of generating polyunsaturated fatty acids (PUFAs), detected in genera *Plesiocystis*, *Enhygromyxa*, and *Haliangium*. Within the last 25 years, myxobacteria have established themselves as front runners in microbiological research and worthy enough to be a thrust area of research in pharmacology for novel bioactive secondary metabolites.

7.2 Discovery and Research

About 50 myxobacterial species have been recognized in the soil habitat and from the bark of the trees. As per the sequence of 16S rRNA, the evolutionary origin of the myxobacterial species lies within the delta subgroup of *Proteobacteria*. Within *Proteobacteria*, only these species have the capability to produce multicellular fruiting bodies giving them a unique niche in the order. *Myxococcus xanthus* shows the capacity of predation to other bacteria with another δ-proteobacterium and *Bdellovibrio bacteriovorus* (Kaiser et al. 2010). First myxobacteria strain was discovered by H.F Link in 1809 and named as *Polyangium vitellinum.* Fruiting bodies produced by *Polyangium vitellinum* were called as *Gastromycetes*. After this, two more species *Stigmatella aurantiaca* and *Chondromyces crocatus* were discovered by M.J Berkeley in 1857 and grouped into *Hyphomycetes*. In 1892 Roland Thaxter pioneered the morphological study and identified these species as myxobacteria. He first described the multicellular behavior of myxobacteria, aggregation of the cell to form a swarm, and process of formation of fruiting bodies. After a broad study on cultivation, Dworkin and Gibson introduced the developmental biology on myxobacteria, whereas Kaiser and coworker focused on molecular genetics. Discovery of biological active secondary metabolites produced by them was initiated by Reichenbach and coworkers and opened a new field on myxobacteria research. At present researchers are primarily focusing on myxobacteria secondary metabolites, developmental biology, and molecular biology (Dawid 2000).

7.3 Distribution and Ecology

The habitat of myxobacteria species is organic-rich soil having pH 5–8. They normally reside in the soil comprised of dead decaying materials, on tree barks, and on dung of herbivores. Only a little information about the cell density is recorded. Approximately, 2000, 4000, 80,000, and 450,000 cells of myxobacteria have been counted in 1 gram of soils of various origins. Fruiting bodies of myxobacteria in soil may consist of 10^3 to 10^6 myxospores. *Nannocytis exedens*, *Corallococcus*

coralloides, *Sorangium cellulosum*, *Myxococcus fulvus*, *Cystobacter* species, and *Myxococcus stipitatus* are few examples of myxobacterial species that reside in soil.

The distinctive myxobacteria species living on the tree bark are *Stigmatella aurantiaca*, *Corallococcus coralloides*, *Myxococcus fulvus*, and different *Haploangium* species. *Chondromyces* and *Stigmatella* species commonly prefer rotting wood. *Myxococcus fulvus*, *Corallococcus coralloides*, *Cystobacter ferrugineus*, *Stigmatella erecta*, *Myxococcus virescens*, *Myxococcus xanthus*, and *Cystobacter velatus* are generally present on the dung pellets of herbivores. Myxobacterial cells usually prefer mesophilic soil with optimum temperature of 30 °C. However, *Polyangium* and *Nannocystis* were found in Antarctica soil and are able to grow in the temperature range of 4–9 °C.

7.4 Cell Structure

Myxobacterial cell is generally 0.6 μm wide and 7 μm to 20 μm long. Vegetative cells are found in two unusual forms:

Cell type I: It is frequently cylindrical with stretchy rods up to 1 μm thick and 20 μm long.

Cell type II: These cells consist of rigid cylindrical rods with rounded ends. They are 1 μm wide and 10 μm long.

Myxobacterial cell wall, like other gram-negative bacteria, consists of peptidoglycan. Outer membrane encloses proteins, phospholipids, and lipopolysaccharides. Unlike most eubacteria, they contain galactose and glucosamine besides mannose. Ketodeoxyoctanoic acid is present in their lipopolysaccharides.

In the cytoplasm, mesosomes, different intracellular granules, and inclusion bodies have been found. Microtubules and contractile fibrils are involved in the gliding movement of vegetative cells. Lunsdorf and Reichenbach observed a structure consisting of rings and elongated components below the outer membrane. These components are liable for the gliding movement of myxobacteria, by developing a wave-like motion that helps cells in gliding. Slime excreted during the movement is expressed as a complex of protein-polysaccharide-lipid components. This complex helps in the proteolytic activities of myxobacteria via degrading the protein of the other cells.

Myxococcus xanthus has pili and fimbriae on the surface of the cells which are involved in interactions of the cells. They also play an important role in cohesion of the two different cells and social behavior of myxobacteria. The pili are arranged in polar orientation, whereas fimbriae are peritrichous (Dawid 2000).

Table 7.1 Taxonomical classification of myxobacteria (Dawid 2000)

Order	*Myxococcales* (the myxobacteria)	
Suborder	*Cystobacterineae*	
Families	*Myxococcaceae*	*Cystobacteraceae*
Genera (number of species)	*Myxococcus* (4)	*Archangium*
	Corallococcus (3)	*Cystobacter* (5)
	Angiococcus (1)	*Melittangium* (3)
		Stigmatella (2)
Suborder	*Sorangineae*	
Families	*Polyangiaceae*	*Nannocystaceae*
Genera (number of species)	*Polyangium* (7)	*Nannocystis* (2)
	Haploangium (2)	
	Chondromyces (5)	
	Sorangium (3)	

7.5 Taxonomy and Phylogeny

Out of 47 myxobacterial species, 19 genera are grouped into suborders *Cystobacterineae*, *Nannocystineae*, and *Sorangiineae*. Classification based on the presence of straight-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) and 16S rRNA comparison are decisive factors of phylogenetic tree of myxobacteria. 16S r-RNA genes reveal a distinct taxonomic group of myxobacteria that are closely related to *Bdellovibrio* and sulfur-metabolizing bacteria. *Sorangiineae* family mostly contains more quantity of SCFA (57–84%) than BCFA, whereas *Cystobacterineae* family revealed the higher BCFA content (53–90%). In *Nannocystineae* family, the proportion of BCFA and SCFA depends on the taxonomic group. Such specific and predominant fatty acids act as biomarker for the identification and classification of myxobacteria. *Nannocystineae* family is remarkably unique for the absence of hydroxy fatty acids (Table 7.1 and Fig. 7.1).

7.6 Genome Analysis

Myxobacteria have a circular genome of 9450 kb which is about double the size of *E. coli* bacterial DNA. The DNA is found to be rich in GC content which varies from 64% to 72%. In *Myxococcus xanthus* (Fig. 7.2), it is 65.5% as revealed by hybridization using *Ase1* restriction enzyme. In suborder *Sorangineae* GC content is more (70–72%) as compared to *Cystobacterineae* (64–70%). Genomic library of *Myxococcus xanthus* has been organized in YAC from SpeI-digested chromosomal DNA (Dawid 2000).

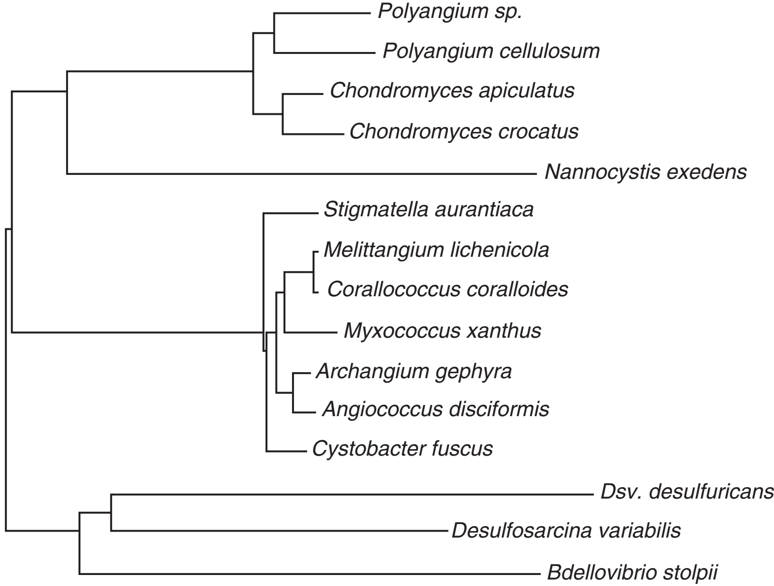

Fig. 7.1 Phylogenetic tree of myxobacterial species (Dawid 2000)

Vulgatibacter incomptus DSM 27710 is the smallest member of the order *Myxococcales* with a genome size of 4.35 Mb, followed by *Anaeromyxobacter* having genome size of ~5 Mb. However, the *Mycobacterium Sorangium cellulosum* has genome size of about 14.78 Mbp and is one of the largest genomic DNA among the bacterial clade known till date. Increase in genome size increases the complexity which is influenced by environmental factors and genetic events such as duplication and insertion of foreign genes. Duplication of proteins found in myxobacteria DNA helps to adapt diverse habitat and complex life cycle.

Myxococcus xanthus and *Stigmatella aurantiaca* species of myxobacteria are the first two prokaryotes which consist of a new class of retro elements called "retron" which codes for reverse transcriptase enzyme like that of retroviruses. Retrons also code for a remarkable satellite DNA which is produced as multicopy single-stranded DNA by reverse transcription process. Extrachromosomal DNA of myxobacteria contain resistance factor which is transferred by conjugation. By using *E. coli* phage, it is possible to integrate the extraneous plasmids into myxobacterial genome and express them.

7.6.1 Whole Genome Sequencing and Annotation of *Myxococcus hansupus*

Myxococcus hansupus consists of single circular chromosome of 9,490,432 nucleotide sequences in which 69.2% of GC content is present. Genome analysis revealed 4 rRNA operons and 67 aminoacyl-tRNA synthetase gene for synthesis of

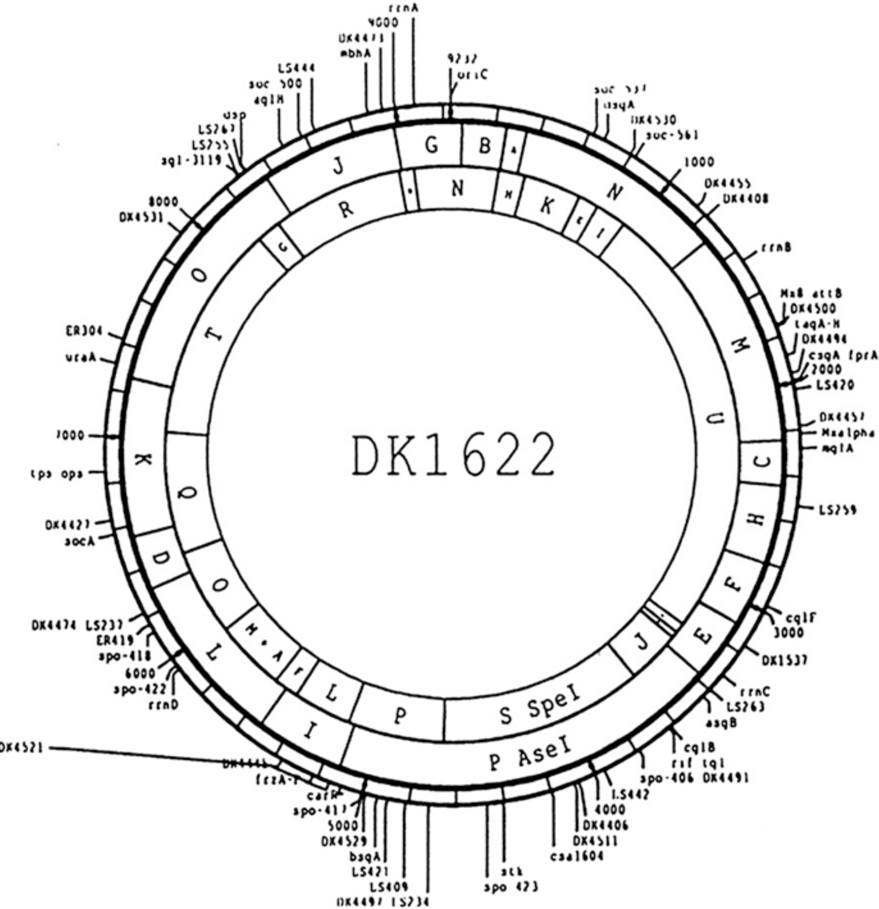

Fig. 7.2 Genetic map of *Myxococcus xanthus* genome (Dawid 2000)

all the 20 amino acids. Within the whole genome, 88.87% genomic density is found to be coding (Fig. 7.3).

In *Myxococcus hansupus*, replication site was recognized at 8,613,829–8,614,077 bp, and dnaA gene was located downstream at a position within 8,646,592–8,645,240 bp. Methylome analysis revealed m6Abased methylation in conserved motif of CTACNNNNNNTGG (79.2%), and CCANNNNNNGTAG (78.1%) at 3rd position CCAAGGC (82.4% motifs) at 4th position WCCCGCAWG motifs (45.2%) and SCCCGCA (53.3% motifs) present at 7th position of the motif region and GATC (31.9% motifs) are found at 2nd position (Table 7.2). m4C methylation was observed in GCGSYDTY (in only 8.3% motifs) at cytosine 2. The increase in protein content of order *Myxococcales* from 7400 to 8200 as compared to 4000 to 5000 in non-*Myxococcales* δ-proteobacteria is involved in the structural regularity functions. *Myxococcus hansupus* shows maximum resemblance with

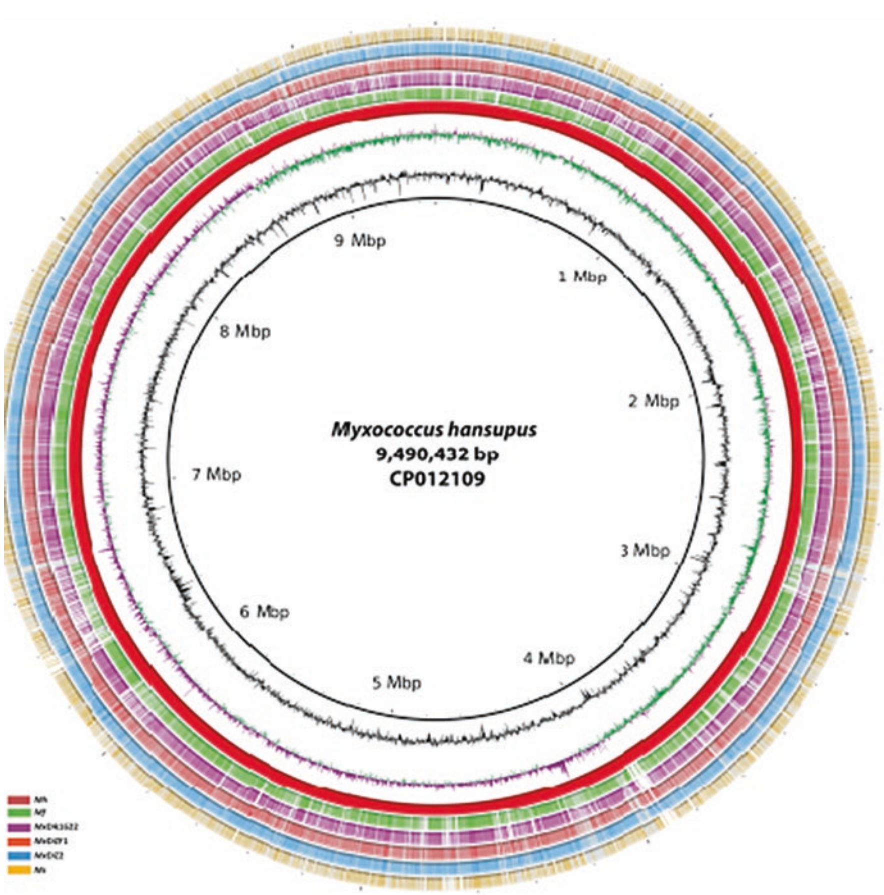

Fig. 7.3 Genetic map of *Myxococcus hansupus* (Sharma et al. 2016)

Myxococcus fulvus (99.45%) and secondly with *Myxococcus xanthus* (99.24% similarity) and then *Myxococcus stipitatus* (98.28% similarity). Its genome shows lowest intergenic distance with *Myxococcus fulvus* genome and highest DNA-DNA hybridization (DDH) value. Bearing the highest DDH value (62.1%) with *Myxococcus fulvus*, *Myxococcus hansupus* is considered as a novel species in *Myxococcus* group, because it shows less than 70% DDH value (Sharma et al. 2016).

7.6.2 Pan Proteome Analysis

The sum of all the protein components related with more than two species is known as pan proteome, and it consists of three different proteomes: core proteome, dispensable proteome, and unique proteome. The pan proteome of six *Myxococcus*

Table 7.2 Assembly statics of *Myxococcus hansupus* (Sharma et al. 2016)

Organism name	*Myxococcus hansupus*
Sequencing data	PacBio P6C4 chemistry sequencing
Total reads	145,073
No. of bases	1,556,757,303 bp
Mean read length	10,730 bp
Average reference coverage	138.05X
Bio-project number	PRJNA161709
NCBI accession number	CP012109
Genome size	9,490,432 bp
GC content	69.2%
Chromosome	1
CDS	7753
% coding density	88,87
CDS from (+) strand	3909
CDS from (−) strand	3844
Max. CDS length	32,543 bp
Mean CDS length	1.088 bp
Hypothetical proteins	2600
Hypothetical proteins%	36.11
tRNA	79
Gene with Pfam domains	5409(69.77%)
Gene with COG domains	5650(72.88%)
Gene with TIGR domains	3686(47.54%)

genomes consists of 46, 392 proteins with 7901 orthologous protein cluster. The proportion of proteins in cluster of different species is 82.09% (*Myxococcus fulvus*), 94.90% (*Myxococcus xanthus*), 66.44% (*Myxococcus stipitatus*), and 81.39% (*Myxococcus hansupus*). Around 4693 clusters of proteins are conserved in every genome of the myxobacteria and defined as core proteome for *Myxococcus* strains. Core proteome includes all the genes that are required for homeostasis and housekeeping functions (that maintain morphological, physiological, and developmental features of microorganisms). Housekeeping functions are performed by 45% of the core proteins that perform cell wall and cell membrane synthesis, amino acid transport, translation, lipid transport, replication, carbohydrate transport, cell motility, etc. Dispensable proteins include proteins that share orthology between two or more genomes but not in all genomes of the ecosystem. These proteins are involved in species-specific functions. Protein consists of gene that develops species level diversity and regulates accessory functions (Sharma et al. 2016).

Unique proteins or distinctive proteins are those which are present only in one genome with no homology with other genomes and were recognized by BLAST analysis. In *Myxococcus* genus, distinctive proteins differ from 12 to 1929 sequences. These unique proteins make a high genome density within the same genus with unknown function (Fig. 7.4).

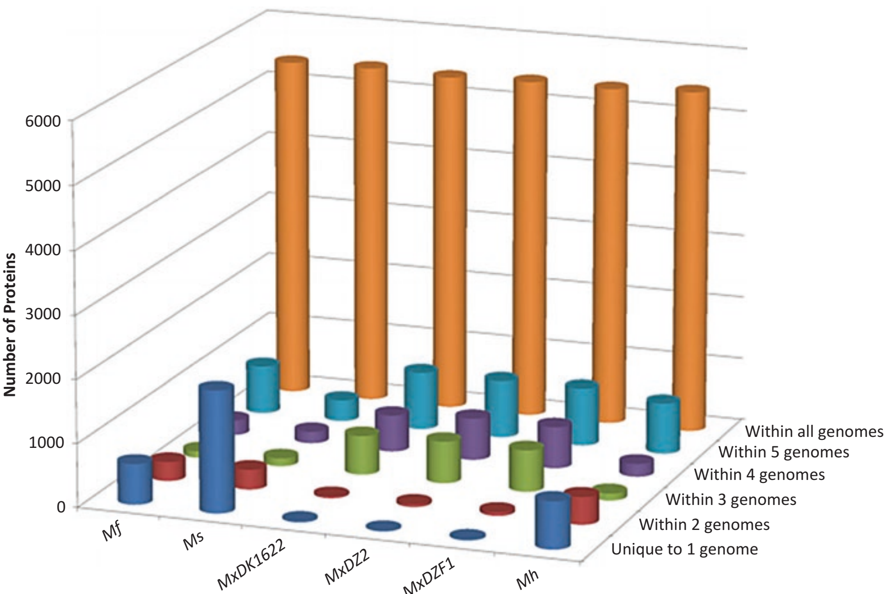

Fig. 7.4 Homologous protein distribution within the genome of *Myxococcus hansupus* (Sharma et al. 2016)

7.6.3 Protein Clustering

Protein clustering analysis suggested that six strains of *Myxococcus* genome consist 590–660 protein clusters, expressed as multiple copies of the same protein because of duplication. Study reveals that in *Myxococcus* genome, 31.33% of proteins are found in the multiple forms. Maximum duplication is represented by protein kinase ABC transporter, long sequence fatty acid CoA ligase, and regulatory proteins of transcriptional process. Pfam clans like protein kinase domain, peptidase_PA.AB_ hydrolase, and thiolase are overexpressed in *Myxococcales* as compared to other *Deltaproteobacteria*. Pfam clans of *Myxococcus* genome characterized the genome expansion of *Myxococcus* species and help these organisms to survive in diverse habitat and form a complex life cycle (Sharma et al. 2016).

7.7 Motility and Swarming Behavior

A characteristic feature of myxobacteria is swarming behavior. Spreading of multicellular cells into unoccupied area is called swarming. The swarming behavior is observed by increasing the colonial diameter. It is because of motility of single cell as compared to the multiplication of the cell. Gliding activity of the cells is dependent on the motility of the cells. Movement of the cells is examined by temperature change, nutrient, concentration, and density of the cells. Vegetative

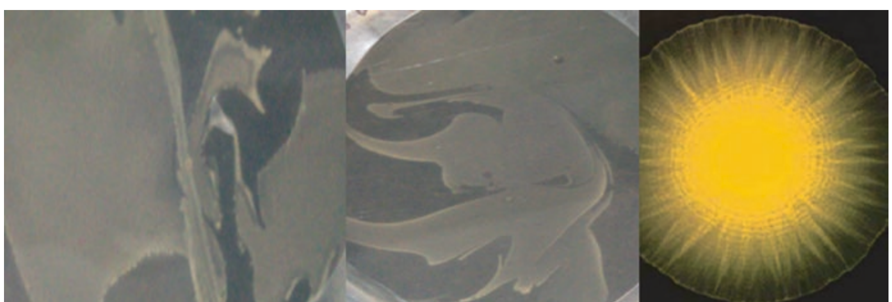

Fig. 7.5 Swarming colonies of *Myxococcus xanthus* on solid agar surface (Kaiser et al. 2010)

swarm formed by *Sorangineae* family shows dense stuffed edges and deep tracks and grooves toward inside. On the other hand, *Cystobacterineae* has flat and thin-layered swarming colonies. Edges of the swarms show peninsula of the cells. The structure of swarming bodies is a specific feature of myxobacteria for putative identification of myxobacteria culture (Dawid 2000).

Myxobacteria show gliding movement on the solid surface as well as water in air interface. This motility is caused via cell bending and due to slime secretion on the solid surface, not because of motility organelle such as flagella. Gliding speed is observed to be 10–60 μm/min. Several theories and models have been projected to elucidate the mechanism of motility in myxobacteria. Per Burchard gliding mechanism is based on the filaments bundle and tubules that are arranged under the cell membrane. Another model stated that conformational change in chain like structure induces a progressing wave on the surface of the cells and causes the movement in the cells (Fig. 7.5).

Gliding behavior is maintained and organized by three independent multigene systems: "A" system involves 22 gene loci and maintains the motility of single cells. Second system is "S" system that consists 10 gene loci and regulates the group of cells. The third multigene system is called "Frizzy" system that controls the reversal behavior of the cells.

7.7.1 *Myxococcus xanthus*: A Model for Motility Study

Cooperative predation and multicellular development of *Myxococcus xanthus* highlight the social behavior of myxobacteria. Coordinated movement uses two motility systems: individual motility or A-motility (adventurous-motility) and grouped motility (social-motility). In the existence of nutrient, the cells show coordinated motility and form a biofilm known as swarm. During predation swarming, colonies cover their prey and lyse cells by hydrolyzing the extracellular biopolymers by exoenzymes. Under starvation conditions, cells move in clusters and initiate the developmental process to exchange extracellular chemical and physical signals to form fruiting bodies. These multicellular fruiting bodies consist of environmental resistant myxospores.

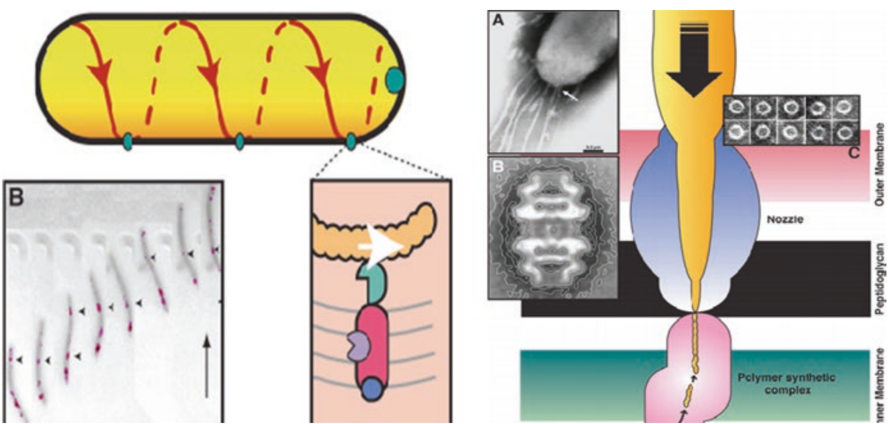

Fig. 7.6 Focal adhesion model and slime secretion model for A-motility (Mauriello et al. 2010)

7.7.1.1 A-Motility (Adventurous-Motility)

A-motility is exemplified by the progress of single cell at the swarm boundary. Cells with A-motility move slowly to explore the fresh environment which alters their route via reversal event by secreting extracellular matrix slime, followed by other cells. A-motility mechanism has been explained by two main theories. First is "helical motor" model also known as "crawling snail model" considered that gliding motors are driven through protein motive force (PMF) and intact MreB cytoskeletons. Second model is "focal adhesion" mechanism. As per this mechanism, the membrane and periplasmic-space components make a complex, called the Glt complex. The Glt complex is linked with the proton motif force of the AgI complex. The AgI-Glt apparatus thus formed, moves along a helical path within the cells. The AgI-Glt apparatus is associated with the substrate by slime secretion that forms a "focal adhesion" site that propels the cells forward (Mauriello et al. 2010).

Extracellular material (ECM) slime in A-motility facilitates the cell adhesion. During single-cell motility, the slime gets deposited in traces, and cell gets to migrate through the trace and support the arrangement of dense region to favor the cell-cell interaction. Ducret et al. (2013) have recommended that slime entrenched in outer membrane material contains signals that promote specific recognition and help in colony organization (Fig. 7.6).

7.7.1.2 S-Motility (Social-Motility)

S-motility, also, twitching motility, is described by the swarming motility of grouped cells and is encouraged by cell-cell interaction. Extracellular matrix (ECM) slime, lipopolysaccharide coating around the cell surface, type IV pili, and extracellular components are associated with S- motility mechanism. Fibrils are the part of ECM which is thick having flexible structure and consist of small fraction of proteins and exopolysaccharides (EPS) that involve glucosamine, galactose, rhamnose, and xylose. EPS show lubricating properties that promote the force generation for

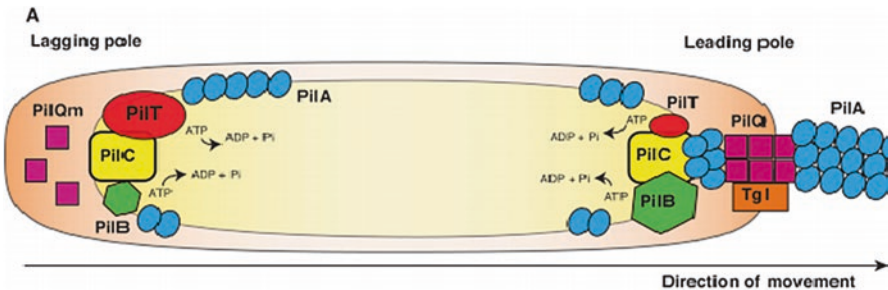

Fig. 7.7 Components of S-motility apparatus (Mauriello et al. 2010)

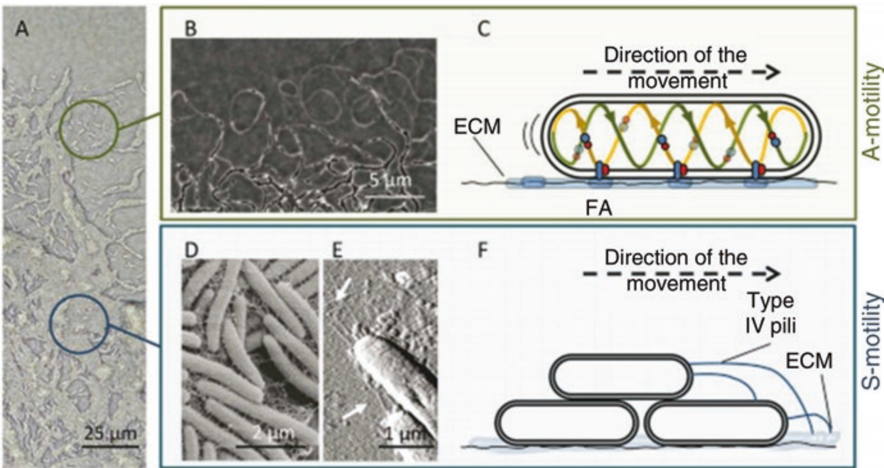

Fig. 7.8 A- and S-motility in *Myxococcus xanthus* (**a**) upper circle shows cells with A-motility, bottom circle, group of cells with S-motility. (**b**) A-motility trail observed under phase contrast microscopy. (**c**) Focal adhesion model of gliding motility. (**d**) Network of the fibril components that maintain cell adhesion. (**e**) Atomic force microscopy of T4 present at the cell pole. (**f**) S-motility model (Muñoz-Dorado et al. 2016)

coordinated cell motility (Figs. 7.7 and 7.8). EPS is observed to provide chemical signal to guide the motility of two systems. It propels the cells by cycles of expansion, addition, and retraction (Muñoz-Dorado et al. 2016).

By performing several experiments, it has been observed that there are five classes of mutation that can affect the gliding movement of myxobacteria. These include A and S genes, mgl (mutual gliding), frz (frizzy), and dsp (dispersed). Mutation in the mgl results in complete loss of motility. Mgl consists of two forms: mglA and mglB. Amino acid sequence of mglA shows similarity with GTP-binding protein, which plays a role in signal transduction, whereas amino acid sequences of mglB show similarity with calcium binding of yeast calmodulin. Mutation in mglB gene reduces the concentration of mglA proteins (Dworkin 1996).

7.8 Fruiting Bodies and Myxospores

Most interesting and conspicuous property of myxobacteria is to form fruiting bodies. These fruiting bodies may appear to be different from globular to bizarre shapes that consist of soft mucous layer and hard slime layers. They consist of myxospores inside the sporangioles that may be present singly or in groups. The size of the fruiting bodies varies from 10 to 100 μm with various colors (Fig. 7.9). Shape, size, and color of fruiting bodies are considered as characteristic features for specific determination (Dworkin 1996).

Fruiting bodies are formed under conditions of nutrient insufficiency and are maintained by concentration of the nutrients, pH of the medium, and temperature. Process of fruiting body formation is a cooperative morphogenesis of swarming cells. Fruiting body formation can be facilitated by cell signaling and A-signal. Response to A-signal indicates the higher concentration of the cells to produce fruiting bodies. First sign of fruiting body production is observed after 4 h of nutrient depletion, when cells start to cumulate and reach to aggregation center within 6–12 h. At this stage, cells show three highly characterized changes which include formation of intracellular lipid bodies, formation of rounded pre-spore cells, and formation of spore envelope. Aggregation process is completed in 24 h, and every new fruiting body contains approximately 10^6 cells. Inside the fruiting bodies, rod-shaped vegetative cells undergo morphological changes to form myxospores. Spores get mature in 72 h after the beginning of starvation process. All the myxospores are

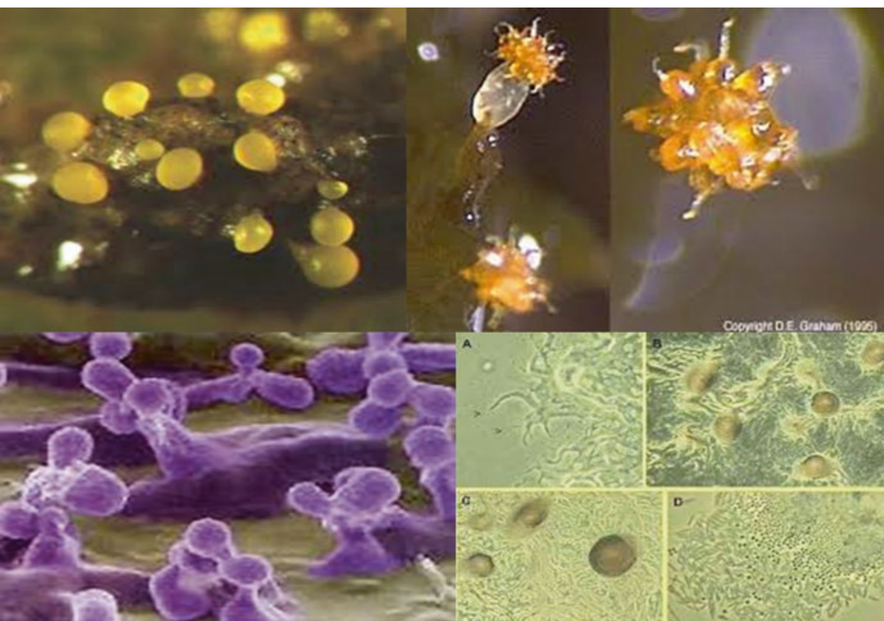

Fig. 7.9 Fruiting bodies of different myxobacterial strains with different colors (Dawid 2000)

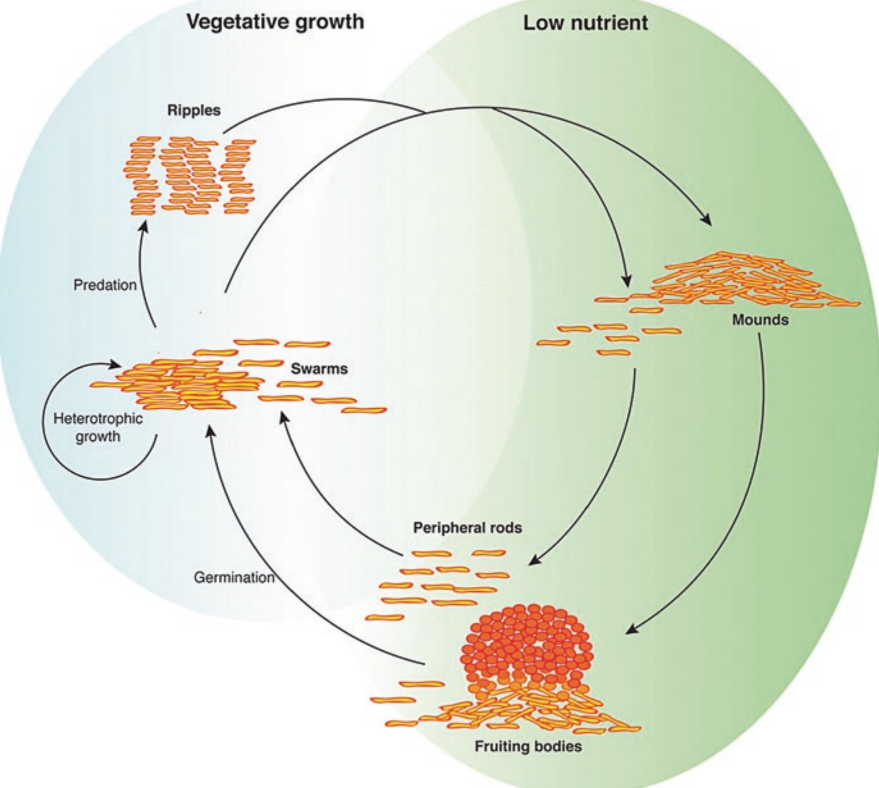

Fig. 7.10 Life cycle (vegetative growth) on solid surface and formation of fruiting bodies in low nutrient condition (Mauriello et al. 2010)

associated with each other by cohesive ECM so as on germination form a new swarm (Fig. 7.10).

Conversion of vegetative cells into myxospores involves remodeling of the cell envelope, synthesis of outer covering of the spore, and formation of two DNA complementary strands and spore-specific lipid components. These lipid components are completely used up during spore maturation.

Peripheral rod cells are population of other cells that are present between fruiting bodies for exploration of nutrients. These cells take advantages of low nutrients that are insufficient to promote further growth and provoke germination of spores. They are differentiated from vegetative cells based on hyperpiliated appearance which do not accumulate extracellular appearance. Peripheral cells are unable to synthesize lipid bodies, because of which, they do not form spores via formation of fruiting bodies. One model explained the cause of peripheral cells that suggest that they are produced when unable to form contact with each other to substitute the C-signal.

Third type of cells that is involved in the spore development is PCD. Finally, the sporulation process involves programmed cell death (PCD). Sporulation happens by two modes of PCD. One of these involves specialized lipids such as phosphatidylethanolamine, which help in increasing cell-permeability. It leads to destruction of the cells. The other mechanism supports the toxin and antitoxin system containing MazF (toxin) and MrpC (antitoxin). Phase variation and cell clustering are two other processes that play role during fruiting body formation. *Myxococcus xanthus* undergo phase deviation process which forms non-yellow color colonies and affects swarm formation, pigmentation of fruiting bodies, and sporulation. Cell cluster population represents the source of heterogeneity; it consists of cells that are close associated within the swarm. During the developmental stage, these cell clusters provide a platform to aggregate the cells and form fruiting bodies. It has been studied that more than 2000 genes are expressed in developing cells. Some scientists have studied the gene network regulatory process which includes three different modules. The first module involves the cascade of enhancer-binding protein (EBP). The second module depends on the EBPs and the A-signal. This module includes mrpAB operon system and mrpC genes so known as mrp module. The third section is FruA module that depends on mrpC and C-signal. In FruA unit, MrpC provoke the expression of fruA gene and act as an antitoxin to organize function of PCD cells. FruA and MrpC2 along with C-signal are involved in the expression regulation of dev operon, which plays a role in sporulation process.

The intercellular signaling involves five signals: A, B, C, D, and E. All these signals are essential for the completion of developmental cycles. A-signal consists of peptides and amino acids. Five genes, *viz.* AsgA, AsgB, AsgC, AsgD, and AsgE are involved in the genomic operon to produce A-signal. In the presence of sufficient population density and nutrients, A-signal prevents the entry of the cells into development cycle. It ensures synthesis of new proteins which are necessary for fruiting bodies and spore before the depletion of nutrients. Nutrition depletion is examined by histidine kinase AsgA which initiate AsgB phosphorylation and express the gene involved in A-signaling. C-signal coordinates the aggregation of the cells and involves the gene csgA, which encode a 25 kDa protein. These proteins remain attached to outer membrane which is processed by protease PopC and function as C-signal. C-signal triggers three process: first is rippling, second is aggregation, and last is sporulation. C-signal molecules increase as the developmental progress by positive feedback mechanism enhances the expression of csgA gene. The initial concentration of C-signal is low and increases gradually. Nutrient deprivation at this point, causes rippling. By the end of 18 h, hundreds of C-signal molecules accumulate and lead to formation of mycxobacterial aggregates. Sporulation is induced at the peak of C-signaling, which is only achieved inside the fruiting body.

7.9 Genome and Proteome of Myxobacteria for Its Multicellularity

Information on 25 myxobacterial genomes is available in the NCBI database. Except *Anaeromyxobacteraceae* family which is found not to form fruiting bodies, myxobacteria have multicellular behavior, so they have large genome and proteome

for the way of life. Transcriptomic analysis of *Sorangium cellulosum* at different pH revealed that it consists of a complex expression pattern and the largest bacterial genome under variable environmental conditions. This bacterium has undergone an unusual genome extension via transfer of genes and their duplication. Myxobacterial genome expansion has arisen mostly by duplication of specific genes that are involved in cell signaling and play role in interaction of the cells and control the multicellularity of the cells. Goldman has observed 1500 gene duplication that occurs in multicellularity transition, whereas metabolic process attains horizontal gene transfer.

Comparative study of genome of four developing and one non-developing myxobacterial strains *Myxococcus xanthus*, *Stigmatella aurantiaca*, *Haliangium ochraceum*, *Sorangium cellulosum* and *Anaeromyxobacter dehalogenans* was performed in which 1052 genes were found to be present in all five species, equivalent to *Myxococcales* core genome. They are present as special cluster of orthologous group. Five percent genes of these conserved regions are required for signal transduction. Myxobacterial genome is found to be rich in cytoplasmic sigma factors, genes involved in carotenoid production, and homeostasis of metal ions, whereas some contribute in motility and development. About 425 genes have been found to be conserved in developing myxobacteria and code for the proteins necessary for formation of fruiting bodies. In 2014, Huntley analyzed ninety five development-related genes, which turned out to be involved in response-to-stravation as well. RelA is a protein which is found to be conserved in all the myxobacteria. *Anaeromyxobacter dehalogenans* belongs to suborder *Cystobacterineae* and is a highly characterized myxobacterial species. The absence of MrpC/FruA/C-signal transduction pathway in *Anaeromyxobacter dehalogenans* and *Sorangium cellulosum* suggested that myxobacterial genome shows a high degree of plasticity which imparts them the ability to form fruiting bodies.

8.6% of the genome of *Myxococcus xanthus* encodes the enzymes which are involved in secondary metabolism and hydrolysis of peptidoglycan membrane, proteases, and metalloproteases, reflecting the predatory properties. Significant production of secondary metabolites with antibacterial, antifungal, antitumor, cytotoxic, insecticidal, and antiparasitic properties has been isolated from the many strains of myxobacteria including *Myxococcaceae*, *Polyangiaceae*, *Cystobacteraceae*, and *Haliangiace* which are under research for their pharmaceutical and industrial applications. Comparative genome study revealed that the genome of predator species lacks riboflavin compound and synthesis of amino acid, whereas highly developed genes responsible for adhesions molecules, proteases enzymes, and other proteins are used for binding of the predator molecule, processing of the prey, and consuming the prey cells.

7.10 Production of Secondary Metabolites

As we know, myxobacteria are mesophilic microorganism and grown in the presence of oxygen. These organotrophs usually propagate between 9 and 38 °C with a generation time of 4 and 12 h (Dworkin 1996). All myxobacteria have potential to

degrade biological macromolecules. Based on utilization of cellulose and inorganic nitrogen, they are classified into two groups. Group I myxobacteria consist of cellulose-degrading strains and members of Sorangineae, which utilize inorganic compounds as well as cellulose. Group II represents most of the myxobacteria class which are unable to utilize cellulose. They depend on amino acids consisting growth substrate (peptone) along with nitrogen.

Under natural condition, they are observed to feed on other microbes by cell lysis, excrete exoenzymes, degrade intact cells, and hydrolyze starch, xylan, and chitin. This cell lysate is nutritional basis for myxobacteria, so as they have been called as micro-predators (Reichenbach and Höfle 1993). Bacteriolytic activity of myxobacteria makes these eubacteria valuable to antibiotics (Dawid 2000). About 80% of the secondary metabolites are observed to produce by prokaryotes, whereas 20% are produced by eukaryotes (mostly fungi). Myxobacteria are known to produce vast number of bioactive secondary metabolites with antifungal, antibacterial, and antitumor activities. Overall 2150 bacteriolytic myxobacteria strains have been studied through which 55% are known to produce bioactive substances. Some of the strains produce biological compounds, belonging to different class such as polyketides, linear and cyclic peptides, heterocyclic compounds, etc. (Dawid 2000).

Myxobacteria exhibit a promising biosynthetic potential. There are a range of biologically active compounds produced by them, among which major molecular classes of compound are polyketides, cyclic and linear peptides, and heterolytic compounds (Höfle and Reichenbach 1990). Majority of bioactive molecules synthesized by myxobacteria are derived from the metabolism of acetate or amino acids, and the ability to synthesize these compounds varies in different strains.

First chemical structural obtained from myxobacteria named ambrucitin was discovered 20 years ago and observed to be antibacterial, followed by myxothiazol produced by *Myxococcus fulvus* (Dawid 2000). Twenty myxovirescin structural variants formed by *Myxococcus virescens* and more than 50 structures of soraphen produced by *Sorangium cellulosum* have been identified (Dawid 2000);

- Several secondary metabolites have been extracted from myxobacteria. Some examples include myxosporin (from *M. fulvus*), Corallopyronin (from *Corallococcus coralloides*) and Sorangicin (from *Sorangium cellulosum*). These compounds have been reported to have bactericidal activity against gram positive bacteria (see Fig. 7.11).
- Another compound myxovalargin produced by strain *Myxococcus fulvus*, *Corallococcus coralloides*, and *Archangium gephyra* inactivates growth of fungi and yeast and displays antimicrobial activity against gram-negative and gram-positive bacteria. It blocks the protein synthesis in eukaryotes (Fig. 7.11).
- Some strains of myxobacteria produce different electron inhibitors. Myxothiazol is known to obstruct the cytochrome complex in respiratory chain. This compound was obtained in 1980 from *Myxococcus fulvus* strain. Myxalamide found in *Myxococcus xanthus* in 1983 is known to inhibit NADH ubiquinone oxidoreductase. Aurachine in 1987 from *Stigmatella aurantiaca* was observed to exhibit same activity as that of myxalamide. All these compounds also show antifungal

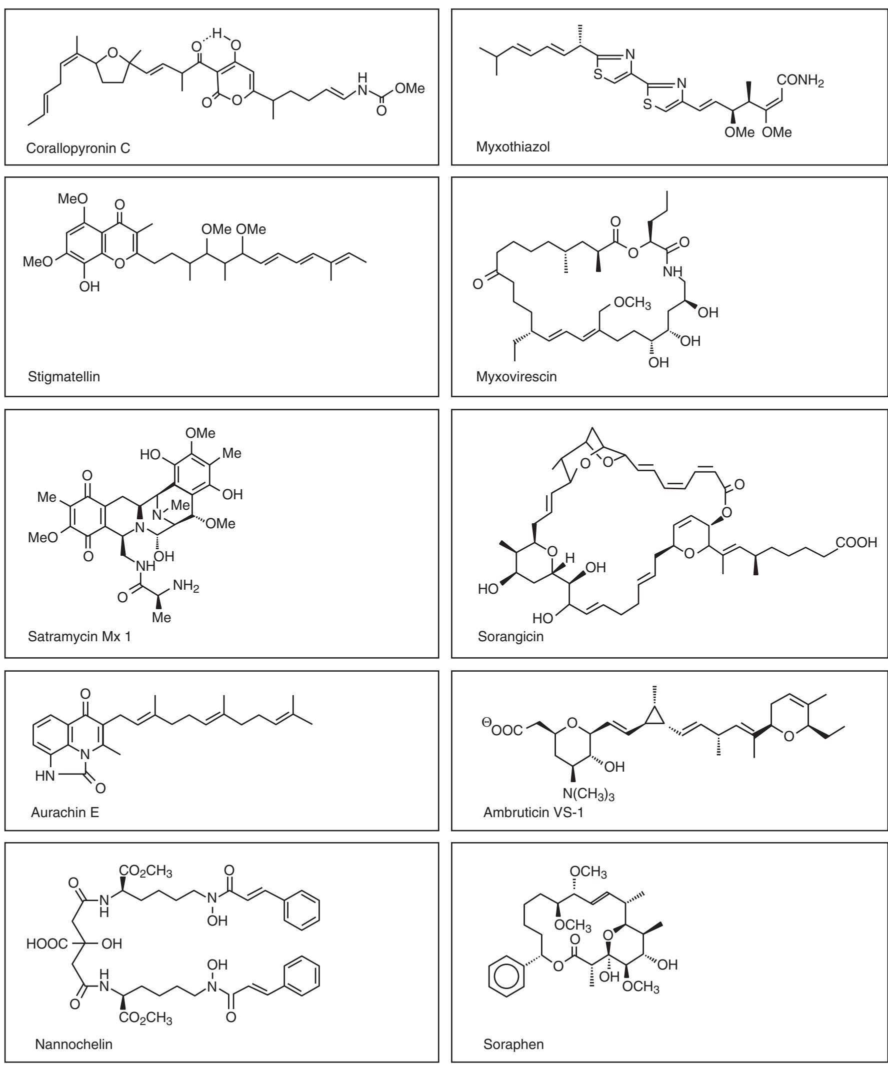

Fig. 7.11 Chemical structure of compounds produced from various myxobacterial species (Kunze et al. 2006; Dawid 2000; Reichenbach and Dworkin (1970)

activity. In 1993 soraphen compound isolated from *Sorangium cellulosum* (Fig. 7.1) acts to block acetyl-CoA carboxylase and is known to act upon phytopathogenic fungi (Dawid 2000).

- In 1987 epothilone (Fig. 7.11) was isolated from *Sorangium cellulosum*, which block cell division and cause apoptosis via acting on cytoskeleton of eukaryotic cells. This compound is considered as antitumor. It has capacity to inhibit growth

of many cancerous cell lines, e.g., breast, intestinal, ovarian cancer (Reichenbach and Dworkin 1970).

- In 2006, Cruentaren, a new antifungal compound, was obtained from *Byssovorax cruenta*, and observed as salicylate-type macrolide (Fig. 7.11) that inhibits mitochondrial ATPase activities of yeast and filamentous fungi (Kunze et al. 2006).

7.10.1 Secondary Metabolites Produced by *Myxococcus xanthus*

Myxococcus xanthus DK1622 is referred to as the best understood myxobacteria and a model strain depicting multicellular differentiation. Eighteen gene clusters have been found in the genome of DK1622 after sequencing (Goldman et al. 2006).

8.5% of total genome of DK1622 is dedicated solely for secondary metabolites, which is much higher than *Streptomyces coelicolor* (4.5%) and *S. avermitilis* (6.6%). Major metabolites produced by DK1622 are myxalamide (Jansen et al. 1983), myxochromides (Meiser et al. 2006), myxochelin (Kunze et al. 2006), myxovirescin (Wenzel et al. 2005), and xanthene (Fig. 7.12) (Gerth et al. 1996).

Myxochelin A

Myxovirescin A1

Myxalamid A

Fig. 7.12 Secondary metabolites produced from myxobacteria species (Dawid 2000; Reichenbach and Dworkin (1970)

7.10.2 Antifungal Activity of Secondary Metabolites Produced by Myxobacteria

Previous studies on 700 strains of *S. cellulosum* have reported that myxobacteria produce some metabolites which have good antifungal activity (Guo et al. 2008), in which the major ones are epothilone, spirangiens, and icumazols. Among these, epothilone shows high toxicity in clusters of animal cells and low antifungal activity. Icumazols like ratjadon possibly interfere with the maintenance of structure of chromosomes. Studies also suggested that epothilone has the potential to be used as antitumor agents.

7.10.3 Antitumor Activity of Myxobacteria Secondary Metabolites

Two strains of myxobacteria identified as *Polyangium vitellinum* and *Sorangium cellulosum* have reported to produce secondary metabolites (Wang et al. 2012). Some of the polyketides, cyclic and linear peptides and heterolytic molecules like disorazole, chondramid, and phoxalone produced by *Stigmatella* WXNXJ-B have also been reported having antitumor properties (Bode et al. 2003).

7.10.4 Steroids Produced by Myxobacteria

Steroids are synthesized in almost all types of eukaryotic cells as per requirement, and they play an important role in the functioning and integrity of the cell. In prokaryotes, this chemical group is generally not synthesized. Microorganisms replace steroids with another class of triterpenoids known as pentacyclic hopanoids. Myxobacterial strains have been reported to produce different kind of sterols, like *Stigmatella aurantiaca* produces cycloartenol (Table 7.3).

Studies have reported a pathway for sterol biosynthesis, and they also suggest that myxobacteria can produce as much steroid as eukaryotes. But, the functional analysis of these chemicals is yet to be initialized to counteract non-availability of the evidences regarding exact function of these sterols. Some scientists think that as the gliding motility of myxobacteria is possible due to the contact between cell and substrate, the fluidity of membrane due to sterols might have an influence on the gliding pattern. *Nannocystis* sp. and *Melittangium lichenicola* process high number of steroid as compared to *Myxococcus* and *Sorangium* sp. (Simkiss and Wilbur 1989).

Table 7.3 Compounds primarily identified from different strains (Bode et al. 2003)

Species	Identified compounds
Nannocytis exedens	Squalene
	Lanosterol
	8(9)-lanosten3β-ol, 8(9)
	24-cholestadien-3β-ol
	8(9)-cholesten-3β-ol
	7,24-cholestadien-3β-7-cholesten-3β-ol
	4,4-dimethylcholestadien-3β-ol
	4,4-dimethylcholesten-3β-ol
	4-methylcholestadien-3β-ol
	4-methylcholesten-3β-ol
Stigmatella erecta	Squalene, cycloartenol
Stigmatella aurantiaca	
Cystobacter ferrugineus	Squalene
C. fuscus	Dehydrosqualene
C. minor	Lanosterol
C. violaceus	Cycloartenol
Polyangium sp.	Lanosterol
Corallococcus coralloides	Squalene
Angiococcus disciformis	Squalene, dehydrosqualene
Melittangium lichenicola	
Archangium sp. *Myxococcus fulvus*	Squalene, dehydrosqualenes

7.11 Biomineralization Potential of Myxobacteria

The ability to precipitate minerals is found in a wide range of organism spread from microscopic creature to chordates. However, there are differences among the process among organisms (Chafetz and Buczynski 1992). Bacteria have been known to possess potential for bioremediation through fractionation, dispersion, or sometimes concentration of the inorganic material (Knorre and Krumbein 2000; Paerl et al. 2001; Jroundi et al. 2007).

Since the early ages, bacteria are using their metabolic activities to change the chemistry of their surrounding environment. Some studies suggest that they contribute to mineral precipitation in both active and passive ways, by helping in mineral deposition.

7.11.1 Production of Mineral by *Myxococcus xanthus*

Thirty percent of all above 60 biogenic minerals are inducible by bacteria, among which more than 16 have been found inducible by the species that come under the genus *Myxococcus*. Some studies strongly suggested that *Myxococcus xanthus* can induce mineralization of phosphate (Gonzalez-Muñoz et al. 1993; Rodriguez-Navarro et al. 2003), carbonates (González-Muñoz et al. 2000; Chekroun et al. 2004), and sulfates (González-Munoz et al. 2003).

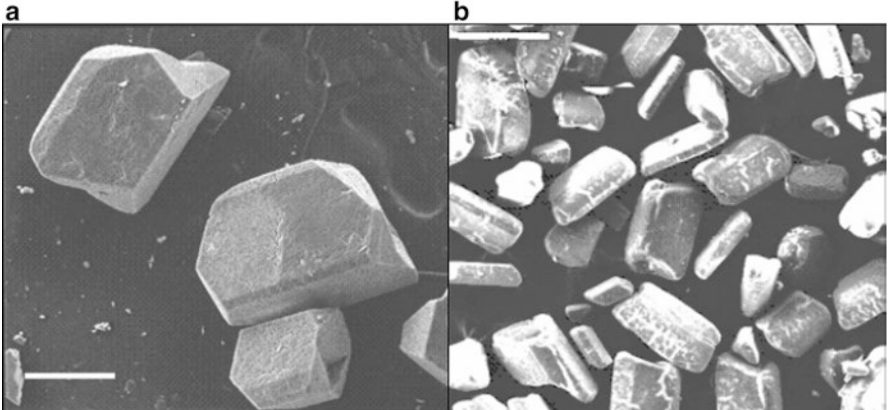

Fig. 7.13 (**a**) and (**b**) SEM photomicrographs of struvite crystals induced by *Myxococcus xanthus*. Bars represent 200 μm (Jimenez-Lopez et al. 2007)

7.11.2 Phosphate Production

Struvite is one of the minerals produced from bacteria which consist of phosphates (Fig. 7.13). Due to its chemical and clinical importance, struvite production has gained attention in various studies (Beavon and Heatley 1963; McLean et al. 1991). Metabolism of nitrogenous compound induces the release of ammonium ions and increasing the pH; these ammonium ions then combine with magnesium and phosphate already present in environment (Robinson 1889; Pérez-García and Rivadeneyra 1989).

Several research groups have studied the production of struvite from *Myxococcus coralloides* and *Myxococcus xanthus* (Omar et al. 1995; Jroundi et al. 2007; González-Muñoz et al. 1994; Omar et al. 1994); these studies have also suggested that there is a wide range of diversity found in crystal morphologies of struvite.

Apart from this, *Myxococcus xanthus* cells have the capabilities to precipitate uranium as meta-uranium, which is uranyl phosphate's mineral phase (Gonzalez-Muñoz et al. 1993). These observations suggest that the strain clearly has an influence on uranium present in the environment wherever it is present (Fig. 7.14).

7.11.3 Carbonate Production

Carbonate mineralization is important for various processes ranging from carbon sedimentation, CO_2 budgeting, to rock and ornamental gemstone formation. (Ehrlich 2006) Bacteria use several processes like photosynthesis, ammonification, sulfate reduction, and denitrification to induce calcium carbonate precipitation. Calcium and aragonite serve as stable polymorphs of calcium carbonates (Riding 2000; Baskar et al. 2006).

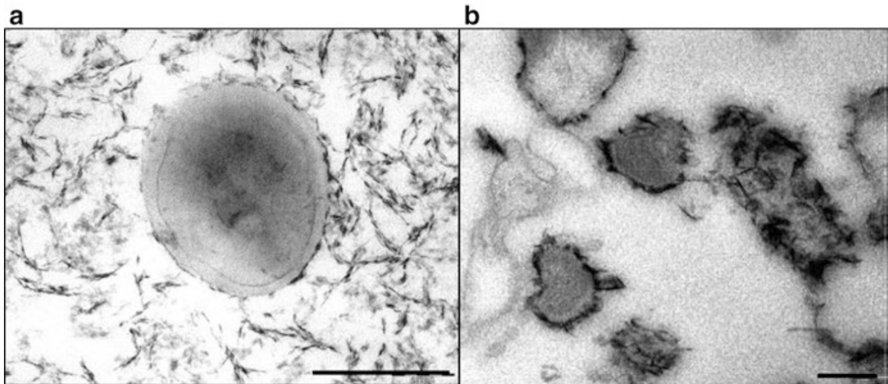

Fig. 7.14 TEM images showing (**a**) accumulation of U (VI) by *Myxococcus xanthus* cells at pH 2.0, (**b**) precipitation of U (VI) as metaautunite induced by *Myxococcus xanthus* at pH 4.5. Bars represent 0.5 μm (Jimenez-Lopez et al. 2007)

Myxococcus xanthus has the ability to induce the precipitation of calcite, Mg-calcite, and vaterite (Fig. 7.15). There is an elevation in alkalinity of the culture media due to release of CO_2 and NH_3 during its metabolic activity.

$$NH_3(g) + H_2O \Leftrightarrow NH_4{}^{+}{}_{(aq)} + OH^{-}{}_{(aq)}$$

$$CO_2(g) + H_2O_{(l)} \Leftrightarrow H_2CO_{3(aq)} \Leftrightarrow HCO_3{}^{-}{}_{(3aq)} + H^{+}{}_{(aq)} \Leftrightarrow CO_3{}^{2-}{}_{(aq)} + 2H^{+}{}_{(aq)}$$

$$Ca^{2+}{}_{(aq)} + CO_3{}^{2-}{}_{(aq)} \Leftrightarrow CaCO_{3(s)}$$

7.12 Concluding Remarks

Myxobacteria, explored from nature, have gained attention worldwide owing to benefits associated with it. It is a unicellular, soil-residing prokaryote which moves and feeds on the predators. *Myxococcus* species form the multicellular constituent known as fruiting bodies and also have spores within which allow them to survive for a longer span of time. The rate of development and sporulation varies as per availability of the nutrient (prey). Nutrient-deficient condition induces development of fruiting bodies, whereas, in the availability of nutrient, the spore germinates and facilitates vegetative growth. During the development, the cell cooperates with each other by the cell to cell soluble exchange along with contact associated with extracellular signals. Cell movement and regulated gene expression are executed by intricate signaling. Myxobacterial biological accomplishment can be estimated as millions of cell are present in a gram of soil, and on the basis of facts, 40 species of myxobacteria can be isolated from the soil from the different parts of the world.

Myxobacteria have received much attention for synthesizing the novel natural products of the low molecular mass of medical importance. Currently, myxobacterial

species are known for their ability to synthesize 5% of known natural products of bacterial origin in greater depth along with diversity among them which is also analyzed. Due to the advent of "genomic era," development in isolation, fermentation, and genetic alteration techniques has enabled us to manipulate myxobacterial strains that aid in advancement in the field of research from the current status. Additionally, the novel strains and metabolites facilitate in delivering novel interactions and enzymatic mechanism; above all the advancement in analytical techniques and novel protocol makes it potent to recognize novel metabolites (even at low concentration). In contrast to 70% of actinomycetes, 18% of fungi, and 6.5% of bacilli, myxobacteria are a better resource of secondary metabolites with its biological activity.

The *Myxococcus xanthus* has emerged as an appealing model for assessing the development of bacteria, surface motility, and cell to cell signaling. The genome size of *M. xanthus* is about 9.14 MB and has duplicate gene along with divergence which contributes to its large genome size. Around 1500 lineage-restricted duplicates were recognized in the genome of *M. xanthus*, representing the coral genes. Nine percent of the genes of *M. xanthus* are found to be associated with the production of secondary metabolites, and these genes are not among the lineage-restricted duplication. On analyzing the prospects, the imminent future of natural product synthesized by myxobacteria seems "promising."

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Part II

Microorganisms for Sustainable Industrial Important Products

Check for updates

Microbial Cellulases: Role in Second-Generation Ethanol Production

8

Anita Saini, Neeraj K. Aggarwal, and Anita Yadav

Abstract

Cellulases are a group of hydrolytic enzymes, which work together as a system, to catalyze the hydrolysis of cellulose. Cellulose is a high-molecular-weight linear homopolymer of D-glucopyranose units linked together with β-(l → 4)-glycosidic bonds, with cellobiose dimer being the repeating unit. In nature, cellulose is present in the plant cell walls, in a matrix of hemicellulose and lignin. Cellulose is the most abundant organic polymer on the earth. The renewability of the cellulosic biomass makes it an attractive feedstock for various industrial applications. Nowadays bioethanol production from cellulose, also known as *second-generation ethanol production*, is the most extensively employed practice, being carried out globally to ensure energy security for future generations by providing a cleaner fuel technology. However, the usefulness of cellulose in ethanol production depends on its conversion into glucose, which can be carried out both chemically as well as enzymatically. Owing to various disadvantages, the chemical methods involving the use of acids are avoided and needed to be replaced with biological methods involving the use of cellulolytic enzymes. Cellulose hydrolysis is mediated by three major types of cellulases, i.e., exoglucanases, endoglucanases, and β-glucosidases. Cellulases are produced naturally by a wide variety of microorganisms, including bacteria, fungi, and actinomycetes. In the second-generation ethanol production, the cost of cellulases is among major economic barriers. However, the exploitation of the microbial cellulolytic systems after their genetic improvement and the other industrially relevant strategies of enzyme production and recycling can make the ethanol production process economical for its wide-scale utilization at the commercial levels.

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Bioethanol production · Cellulases · Lignocellulose · Microorganisms · Second generation ethanol

8.1 Introduction

The emerging issue of energy crisis as a consequence of industrial advancement and urbanization is a major concern worldwide. The extensive exploitation of existing fossil fuel reserves on the earth has limited their availability to meet the energy demands of the increasing world population (Sun and Cheng 2002). The fuels from renewable sources, the *biofuels*, are a promising alternative to this problem. The biofuels are not only the sustainable source of energy but also offer an additional advantage of mitigating air pollution (Chartchalerm et al. 2007). This is because of their potential to cut on net CO_2 emissions due to a closed loop system, in which the rate of CO_2 released equals the CO_2 fixed by the plant during photosynthesis (Robu et al. 2005). Bioethanol is known among most widely used biofuels, especially due to its fuel potential and capability to replace existing transport fuel (Sarkar et al. 2012). In the past centuries, world's bioethanol production relied primarily on *first-generation* biofuel production technology, i.e., the production of ethanol from sugar and starch-based crops. But several concerns such as the land management for the cultivation of edible crops, security of food, and the threat to biodiversity together have led to the development of an advanced technology, i.e., *second-generation* biofuel production from the lignocellulosic biomass (Nanda et al. 2013; Saini et al. 2015a). Lignocellulose is the most abundant biomass on earth, chiefly composed of cellulose, hemicellulose, and lignin (King et al. 2010). The cellulosic component in the plant cell wall is composed of chains of β-1,4-linked glucose units. The enzymatic conversion of cellulose into the fermentable sugar (glucose), the saccharification, is the crucial step in the production of second-generation ethanol (Khare et al. 2015). The hydrolysis of cellulose is mediated through the cellulase enzyme. The high cost of cellulases is one of the prominent challenges in the process of biomass to ethanol conversion (Zhang and Zhang 2013). Several strategies, such as the search for potent microbial cellulases, improvement in microbial cellulases synthesis by optimization studies (Rakshit and Sahai 1991), development of efficient cellulolytic systems by genetic engineering (Yao et al. 2016) or other techniques, and recycling of cellulases (Sun and Cheng 2002) by immobilization (Kumakura et al. 1989), can significantly cut down the cost of the saccharification process. This chapter briefly reviews the microbial sources of cellulases, the hydrolytic mechanism of cellulolytic enzymes, the conversion pathway of cellulose (in lignocellulosic biomass) into ethanol, and the approaches to overcome the economic barrier in the second-generation ethanol production due to the cost of cellulases.

Fig. 8.1 Structure of cellulose

8.2 Cellulose

Cellulose is a water insoluble, high-molecular-weight linear homopolymer composed of thousands of D-glucopyranose units linked together with β-(1 → 4)-glycosidic bonds (Rubin 2008) (Fig. 8.1). The degree of polymerization, however, varies among different sources (Sukumaran et al. 2005). The cellobiose disaccharide makes the repeating unit of cellulose polymer and consists of two glucose units (glucose-β-1,4-glucopyranoside) rotated at an angle of 180° with respect to each other (Horn et al. 2012) (Fig. 8.1). Consequently, the hydroxyl groups on two sides of a chain are distributed evenly thus providing a symmetry to the whole structure. The cellulose chains are held together by various interchain hydrogen bonds. Additionally, the hydrophobic interactions cause the parallel cellulose chains to aggregate together in the form of *microfibrils* (Dashtban et al. 2009). The microfibrils make still larger bundles referred as *fibrils*, which at the next level assemble into cellulose fibers (Brodeur et al. 2011). Thus, a three-dimensional lattice of cellulose is constructed, which can appear in several supra-molecular structures. It, however, predominantly contains amorphous (less ordered) and crystalline (highly ordered) regions (Kumar et al. 2009). The celluloses in various biomass sources vary in their crystallinity, which has a huge impact on the susceptibility of the cellulose to different chemical and enzymatic hydrolysis reactions. The amorphous regions are readily digestible by cellulolytic enzymes compared to the crystalline regions, which require chemical modifications for loosening their structure.

8.3 Cellulases

Cellulases, classified among glycosyl hydrolases, involve a complex of enzymes together referred as to *cellulase enzyme system* which is primarily composed of exoglucanases (EC 3.2.1.74), endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) (Sharada et al. 2013; Dorez et al. 2014; del-Pulgar and Saadeddin 2014). Different cellulases vary in their mechanisms, site of action, and substrate specificities. The exoglucanases (CBHI or

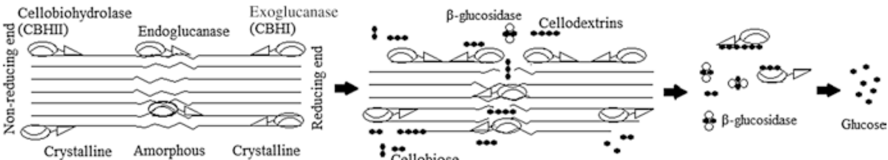

Fig. 8.2 Scheme of cellulose hydrolysis by cellulases. (Adopted from Lynd et al. 2002)

1,4-β-D-oligoglucan cellobiohydrolases) catalyze the removal of glucose units from the reducing or non-reducing ends of the cellulose chains (Kumar et al. 2008). The endoglucanases (endocellulases or β-1,4-endogluconhydrolases, or carboxymethyl cellulases) act randomly inside the cellulose chains; release dextrans or oligosaccharides of variable lengths, while exhibiting cyclic adsorption and desorption; and generate new chain ends in the hydrolysis process (Perez et al. 2002; Sadhu and Maiti 2013; Obeng et al. 2017). They are mostly active in the amorphous regions of the cellulose. The cellobiohydrolases (CBHII or exoglucanases) cleave the glycosidic bonds at non-reducing ends and, thus, release cellobiose units (Lynd et al. 2002; Sadhu and Maiti 2013). The exoglucanases show a distinct feature of the processivity, which enables them to hydrolyze the crystalline regions of the cellulose (Arantes and Saddler 2010). β-Glucosidases (or cellobiases) are required at the final step of the hydrolysis process. They catalyze the hydrolysis of cellobiose units into the glucose monomers (Sun and Cheng 2002; del-Pulgar and Saadeddin 2014). Thus, the complete hydrolysis of cellulose involves a complementary action of all the enzymes of the cellulase complex (Sukumaran et al. 2005). The entire depolymerization process results from the interplay of interactions among these enzymes such as the endo-exo synergy (synergy between endoglucanases and exoglucanases), the exo-exo synergy (synergy between exoglucanases acting on the reducing and non-reducing ends), and the interaction between cellobiohydrolases and β-glucosidases and those between catalytic- and carbohydrate-binding domains (Henrisatt et al. 1985; Lynd et al. 2002; Zhang et al. 2006). Figure 8.2 depicts the schematic presentation of enzymatic deconstruction of cellulose polymer. Furthermore, the exo- and endocellulases are negatively regulated by its end products through feedback inhibition. This mandates the removal of end products in order to hydrolyze the polymer to completion.

The polypeptides of the cellulases fold in the three-dimensional, functionally active enzymes with discrete domains (Arantes and Saddler 2010). The cellulases share some common structural features, i.e., the presence of catalytic domain at the N-terminus; a carbohydrate-binding domain, CBD (also known as a *carbohydrate-binding module*, CBM), at the C-terminus; and a short poly-linker joining these two domains (Zhong et al. 2008; Obeng et al. 2017) (Fig. 8.3). The CBD mediates the binding of the cellulase onto the surface of cellulose and positions the enzyme such that the catalytic domain comes in close proximity to the substrate. The presence of CBMs is particularly important for the initiation and processivity of the exoglucanases (Lynd et al. 2002). The exo-acting cellulases (CBHI and CBHII) have a tunnel-shaped closed catalytic site, while endoglucanases acting on the internal sites have a cleft-shaped open-active site (Arantes and Saddler 2010; Obeng et al. 2017).

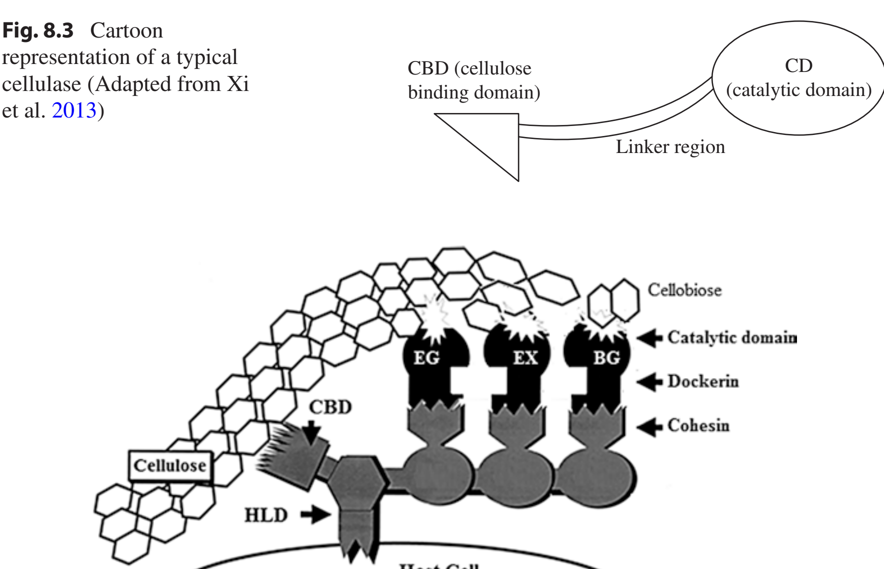

Fig. 8.3 Cartoon representation of a typical cellulase (Adapted from Xi et al. 2013)

Fig. 8.4 Simplified architecture of a cellulosome. (Modified from Doi et al. 2003 and Gilmore et al. 2015) (*HLD* hydrophilic domain, *CBD* cellulose-binding domain, *EX* exoglucanase, *EG* endoglucanase, *BG* β-glucosidase)

The cellulolytic enzymes are found in microorganisms either as the complexed or noncomplexed cellulases (Lynd et al. 2002). The aerobic cellulolytic microorganisms are known to secrete various cellulases extracellularly in the free or noncomplexed forms (Bayer et al. 2004). The complexed systems, known as cellulosomes, are characteristics of anaerobic cellulolytic microorganisms (Doi and Kosugi 2004). The cellulosomes are a complex of multifunctional scaffolding proteins and various enzymatic subunits such as the endoglucanases, cellobiohydrolases, xylanases, and other polymer hydrolytic enzymes (Bayer et al. 2004). The scaffolding proteins consist of "cohesion" domains, which interact with the dockerin protein domains present on each of the enzymatic subunits (Fig. 8.4). The high-affinity interactions between the cohesion and dockerin proteins govern the assembly of a cellulosome. Mostly the binding of the cellulosome with the cellulosic substrate is mediated through a scaffolding-borne cellulose-binding module (CBM or CBD) which is a part of the scaffolding subunit (Bayer et al. 2004; Doi and Kosugi 2004; Gilmore et al. 2015). The CBD appears to bind the cellulosome more strongly to the crystalline regions than to the amorphous regions in the cellulose. The cellulosome structural studies have revealed the presence of hydrophilic domains (HLDs) in a number of scaffoldings, which assist in binding the cellulosome to the host cell envelope (Doi et al. 2003). Similar to the free cellulases, the cellulosomal endo- and exo-acting and β-glucosidase enzymes act in synergism for complete hydrolysis of the cellulose polymer (Gilmore et al. 2015).

8.4 Cellulase-Producing Microorganisms

In nature, a wide variety of microorganisms, belonging to the eukaryotic (fungi) and prokaryotic (eubacteria and actinomycetes) groups, are known to produce cellulases (Gautam et al. 2012). Their cellulolytic systems, however, vary from the free form in the aerobes to the complexed cellulosomes in the anaerobes. The cellulose degraders can also be classified among mesophilic and thermophilic groups. Their physiological characteristics determine the type of environment inhabited by them. However, the cellulolytic potential of cellulases from physiologically diverse microbial sources is exploitable in various applications based on their specific relevance. Table 8.1 shows the list of some of the microorganisms known for their cellulase production potential. *Trichoderma reesei* is one of the most extensively studied cellulase producers and is exploited for cellulase production at the commercial levels (Singh et al. 2017).

8.5 Bioethanol Production from Lignocellulose

Second-generation ethanol (also referred to as *cellulosic ethanol*) from the plant cell wall lignocelluloses is a promising alternative to the first-generation bioethanol produced from sugar and starch crops (Kang et al. 2014). Typically, most of the lignocellulosic biomass is comprised of 30–50% cellulose, 15–35% hemicellulose, and 10–25% lignin (Limayem and Ricke 2012). The composition, however, varies with the source of the plant biomass (Table 8.2). Despite this variation, the lignocellulosics consist of substantial amounts of fermentable sugars, C6 and C5, fixed in their cellulose and hemicellulose polymers, respectively. The release of these sugars requires the depolymerization of the carbohydrate polymers of the cell wall.

The conversion of the cellulosic biomass into ethanol can be performed either biochemically or by a thermochemical process (Demirbas 2007). The thermochemical conversion involves the gasification of the biomass at high temperature to produce syngas, which is converted into ethanol chemically using molybdenum disulfide as a catalyst (Rastogi and Shrivastava 2017). The biochemical conversion of the biomass is an eco-friendly method (Sarkar et al. 2012). Figure 8.5 outlines the scheme of ethanol production from the cellulosic biomass biochemically. The bioconversion of the lignocellulose is a complex process because the recalcitrance of the plant cell walls due to lignin hinders in the accessibility of the carbohydrate polymers buried in its interior. Thus, the foremost step in the biochemical conversion is the pretreatment of the biomass, which involves the deconstruction of the cell wall by partial removal of its components, i.e., the lignin, hemicellulose, or cellulose (Sun and Cheng 2002; Kumar et al. 2009). The biomass can be pretreated using different physical, chemical, biological, or combination of these methods (Kumar et al. 2009). Each method has its own advantages and limitations. The suitability of the pretreatment method depends on the type of the biomass feedstock (Khare et al. 2015). The efficiency of the pretreatment step, however, is the key factor determining the yield of the hydrolysis in the cellulosic ethanol production process (Alvira

Table 8.1 List of different cellulase-producing microorganisms

Microorganism	References
Fungi	
Aspergillus niger; A. nidulans; A. oryzae; A. terreus; A. flavus, A. fumigatus; Fusarium solani; F. oxysporum; Penicillium brasilianum; P. occitanis; P. decumbans; P funiculosum; P. fumigosum; P. janthinellum; Humicola insolens; H. grisea; Melanocarpus albomyces; Trichoderma reesei; T. longibrachiatum; T. harzianum; T. atroviride; Chaetomium cellulyticum; C. thermophilum; Neurospora crassa; Thermoascus aurantacus; Mucor circinelloides; Paecilomyces inflatus; P. echinulatum; Coniophora puteana; Lanzites trabeum; Poria placenta; Tyromyces palustris; Fomitopsis sp.*, Phanerochaete chrysosporium; Sporotrichum thermophile; Trametes versicolor; Agaricus arvensis; Pleurotus ostreatus; Phlebia gigantea; Talaromyces emersonii; Anaeromyces mucronatus; Caecomyces communis; Cyllamyces aberensis; Orpinomyces* sp.*; Sclerotium* sp.*; Piromyces* sp.*; Neocallimastix frontalis; I. lacteus; Talaromyces* sp.	Mathew et al. (2008), Pandey et al. (1999), Lynd et al. (2002), Sukumaran et al. (2005), Dashtban et al. (2009), Kuhad et al. (2011), Li et al. (2011), Liu et al. (2011), Mrudula and Murugammal (2011), Carvaloh et al. (2014), Sajith et al. (2014), Imran et al. (2016) and Sohail et al. (2016)
Bacteria	
Bacillus subtilis; B. pumilus; B. amyloliquefaciens; B. licheniformis; B. circulans; B. flexus; B. thuringiensis; B. cereus; Cellulomonas biazotea; Cellvibrio gilvus; Eubacterium cellulosolvens; Geobacillus sp.*; Paenibacillus curdlanolyticus; Paenibacillus polymyxa; Pseudomonas cellulosa; Salinivibrio* sp.*; Rhodothermus marinus; Cytophaga hutchinsonii; Cellvibrio japonicas; Microbacterium* sp.*; Bosea* sp.*; Anoxybacillus flavithermus; Fibrobacter succinogenes; Ruminococcus albus; Caldicellulosiruptor saccharolyticus; Caldicellulosiruptor obsidiansis; Rhodothermus marinus; Pseudomonas fluorescens; Pseudomonas putida; Bacteroides* sp.*; Clostridium cellulolyticum; C. acetobutylium; C. papyrosolvens; C. thermocellum; C. straminisolvens; C. stercorarium*	Lynd et al. (2002), Russell et al. (2009), Kuhad et al. (2011), Sizova et al. (2011), Sadhu and Maiti (2013), Sharada et al. (2013), Imran et al. (2016) and Obeng et al. (2017)
Actinomycetes	
Cellulomonas fimi; C. bioazotea; C. uda; C. cartae; C. cellusea; C. flavigena; C. cellulans; Streptomyces sp.*, S. drozdowiczii; S. lividans; S. flavogrisus; S. nitrosporus; S. nitrosporeus; S. albaduncus; S. albogriseolus; S. reticuli; S. cellulolyticus; S. malachitofuscus; S. glomeratus; S. stramineus; S. actuosus; S. viridobrunneus; S. matensis; S. longispororuber; Thermomonospora* sp.*; T. fusca; T. curvata; Microbispora* sp.*; M. bispora; Thermobifida fusca; Rhodococcus* sp.*; Saccharomonospora* sp.*; Nocardia* sp.*; Thermoactinomyces* sp.	Thayer et al. (1984), McCarthy (1987), Walter and Schrempf (1995), Harchand and Singh (1997), Xianzhen (1997), de Lima et al. (2005), Eida et al. (2012), del-Pulgar and Saadeddin (2014) and Saini et al. (2015b)

Table 8.2 Chemical composition of various lignocellulosic biomass

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Wheat straw	29–35	26–32	16–21	Anwar et al. (2014)
Rice straw	32.1	24	18	Anwar et al. (2014)
Rice husks	28.7–35.6	12.0–29.3	15.4–20.0	Isikgor and Becer (2015)
Oat straw	31.0–35.0	20.0–26.0	10.0–15.0	Isikgor and Becer (2015)
Ray straw	36.2–47.0	19.0–24.5	9.9–24.0	Isikgor and Becer (2015)
Sugarcane bagasse	35.2	24.5	22.2	Rezende et al. (2011)
Switchgrass	35.0–40.0	25.0–30.0	15.0–20.0	Sannigrahi et al. (2010)
Poplar	42–49	16–23	22	Sannigrahi et al. (2010)
Corn cobs	33.7–41.2	31.9–36.0	6.1–15.9	Isikgor and Becer (2015)
Corn stalks	35.0–39.6	16.8–35.0	7.0–18.4	Isikgor and Becer (2015)
Barley straw	36.0–43.0	24.0–33.0	6.3–9.8	Isikgor and Becer (2015)
Hardwood	45–47	25–40	20–25	Kang et al. (2014)
Softwood	40–45	25–29	30–60	Kang et al. (2014)
Grasses	25–40	35–50	10–30	Limayem and Ricke (2012) and Isikgor and Becer (2015)
Agricultural residues	37–50	25–50	5–15	Kang et al. (2014)

Fig. 8.5 Simplified overview of cellulosic ethanol production pathway

et al. 2010) as it ensures the accessibility of the carbohydrate polymers for their subsequent hydrolysis in the fermentable sugars. In the next step, i.e., the saccharification, the celluloses and hemicelluloses are hydrolyzed into C6 and C5 sugars with the help of cellulases and hemicellulases enzymes, respectively (Sarkar et al. 2012). The last step involves the conventional approach of fermentation of C6 and C5 sugars into ethanol using either native or genetically modified ethanologenic microbes (mostly yeast and bacteria) (Gnansounnou and Dauriat 2005).

The biomass saccharification can also be carried out using a chemical method of two-stage acid hydrolysis (Fig. 8.6). In the first stage, the hemicellulose is hydrolyzed to the pentose (C5) sugars by dilute acid hydrolysis. Thereafter, the solid fraction containing cellulose and lignin is separated from the liquid portion. The cellulose in the solid fraction is hydrolyzed to hexose (C6) sugars, using acid at relatively harsher conditions (second stage of acid hydrolysis). The liquid hydrolyzates from the first- and second-stage hydrolysis containing C5 and C6 sugars are fermented to ethanol with the help of pentose- and hexose-fermenting microbes, respectively.

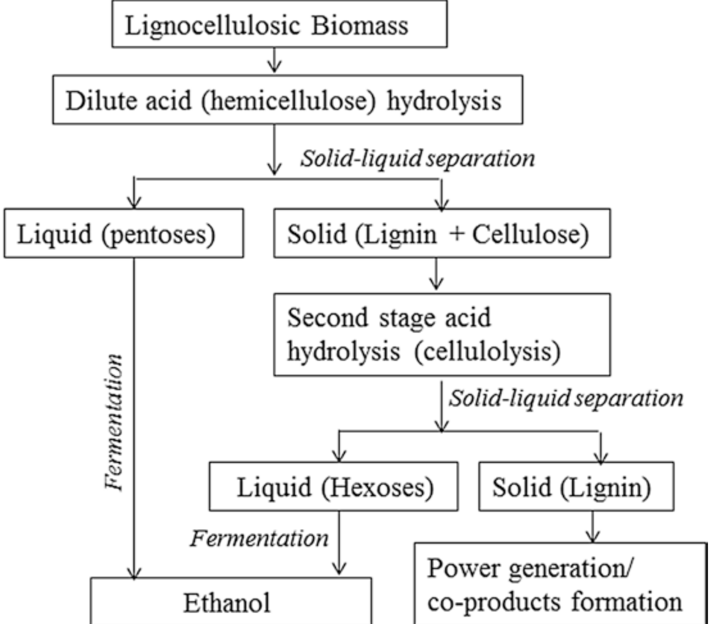

Fig. 8.6 Two-stage acid hydrolysis of the biomass for bioethanol production

Historically, the enzymes have been found to be expensive for the economical production of the fuel ethanol from the biomass (Virkajarvi et al. 2009). Sulfuric acid is less expensive than the cellulolytic enzymes, although disposal costs associated with its use and other problems such as recycling of acid and corrosion of reactors increase the cost of the process significantly. Moreover, the largest drawback to using sulfuric acid is that it readily degrades the glucose itself at the high temperatures (Mosier et al. 2002). The recent studies, on the other hand, suggest that the use of efficient cellulolytic enzymes for cellulose hydrolysis is relatively more economical and environmentally benign approach (Chandel et al. 2007). Nevertheless, the enzymatic hydrolysis of the biomass is the primary interest of the researchers because it offers several advantages over the chemical processes, i.e., the higher yields, higher selectivity, lower energy inputs, and moderate operating conditions (Yang et al. 2011). Table 8.3 shows the enzymatic hydrolysis of various feedstock using commercial cellulases.

8.6 Cost of Cellulases: A Challenge in Cellulosic Ethanol Production

The process of cellulosic ethanol production runs over a few difficulties at different levels in its way to the production of ethanol economically: (1) availability of abundant supply of renewable feedstock, (2) efficient and economical pretreatment technology with minimum carbohydrate loss or inhibitors formation, (3) need for

Table 8.3 Hydrolysis of various feedstock using commercial cellulases

S. no.	Feedstock	Enzyme source	Outcome	References
1	Sugarcane bagasse	Commercial enzyme (Environmental BioTechnologies, Inc.)	41–67% glucose conversion;36–64% ethanol production potential	Kaar et al. (1998)
2	Wheat straw	Celluclast 1.5 L, Novozyme 188, and Lacasse from *Trametes versicolor*	Yielded 565 mg/g of sugars and 13 g/L ethanol	Saha and Cotta (2006)
3	Rice hull	Celluclast, Novozyme, and Viscostar	90% reducing sugar yield	Saha and Cotta (2007)
4	Soyabean hull	Cellulase Spezyme CP, β-glucosidase (Novozyme 188); pectinase (Sigma-Aldrich Corp.) and hemicellulase (Sigma-Aldrich Corp.)	Yielded 25–30 g/L ethanol in simultaneous saccharification and fermentation process	Mielenz et al. (2009)
5	Rice straw	Cellulase (Genencor, Spezyme)	93.1% of theoretical glucose yield	Chen et al. (2011)
6	Rice straw	Cellulase from *A. niger* and xylanase from *T. viride* (100 U/g) (Sigma Chemical Co.)	371.91 g glucose/kg of dry rice straw with 84% hydrolysis yield	Chang et al. (2011)
7	*Eucalyptus globulus*	Commercial cellulase (Celluclast 1.5 L) and β-glucosidase (Novozyme 188)	60% of the theoretical glucose yield	Yanez-S et al. (2013)

cost-effective and potent saccharolytic enzymes, and (4) competent ethanologenic strains with enhanced tolerance (Saini et al. 2015a) Thus, it is clear that the cost of the carbohydrate hydrolases including cellulases is among major contributors governing the economics of the lignocellulosic biofuel production. In fact, the dedicated reports on the economic evaluation of the cellulosic ethanol production process have discussed that the cost of cellulase production is much higher than is proposed in the literature (Klein-Marcuschamer et al. 2012).

8.7 Future Research on Cellulases

8.7.1 Search for Potent Cellulolytic Enzymes

Conquering the obstruction due to the cost of cellulase production and development of more efficient, specific and stable cellulases are the real objectives of future research on cellulases (Sukumaran et al. 2005). The simplest strategy for meeting these objectives is to look for novel microbes with high cellulolytic potential. Though it appears that the search for potent cellulolytic microbes over a couple of decades has already reached a plateau, recent studies have shown that many habitats such as extreme environments and relatively unexplored ecosystems still reserve

many known as well as unknown cellulase producers, which can exhibit high cellulolytic potency as well as specificity. Some of the diverse habitats explored recently for the isolation of novel cellulase producers include hot springs, thermal vents, termite's guts (Acharaya and Chaudhry 2012), deep-sea sediments (Odisi et al. 2012), high-mountain soil ecosystems (Avellaneda-Torres et al. 2014), insects' gut (Haung et al. 2012; Dantur et al. 2015), Antarctica's ecosystems (Lamilla et al. 2017), synthetic extreme environments such as car wash effluent (Sibanda et al. 2017), etc. The microbes from extreme environments are an attraction because of their applicability in the industries at harsh conditions. The thermostability is the most desirable feature of the cellulase enzymes for their industrial application in the bioethanol production, which drives the search for cellulases form thermophilic microbes (Azizi et al. 2015; Li et al. 2011).

Besides, non-culturable microbes are also an excellent source of cellulolytic genes. The researchers have recently broadened their search and started aiming for the non-culturable cellulase producers. The most widely recognized approach involves the construction of a metagenomic library followed by its functional screening (Maki et al. 2009; Yang et al. 2016). The functional screening, however, is a very cumbersome technique as it requires the construction of a huge library to get few active clones. The more advanced techniques such as pyrosequencing followed by sequence screening are a better way of finding cellulases gene in the metagenome (Rooks et al. 2012). A similar approach has been used for obtaining cellulases from anaerobic beer lees-converting consortium, wherein the metagenome was sequenced and then screened for cellulase sequences. Three of the obtained cellulase genes were cloned and expressed in *E. coli* and were found exhibiting considerable cellulase activities (Yang et al. 2016).

8.7.2 Enhanced Synthesis of Cellulases

The enhancement in the production of cellulase enzymes is another strategy being attempted consistently for reducing the cost of the cellulases. The use of the low-cost renewable substrates can significantly reduce the cost of enzyme production. The lignocellulosic biomass can be used as a raw material for the production of enzymes (Klein-Marcuschamer et al. 2012; Ellila et al. 2017). The cellulases are inducible enzymes (Acharaya and Chaudhry 2012). Many of the current commercial cellulase production technologies rely on fungal microorganisms. The research studies have shown that the expression of cell wall-degrading enzymes in the filamentous fungi is best induced in the presence of insoluble lignocellulosic substrates (Znameroski et al. 2012; Sohail et al. 2016). Also, it has been proposed that the enzyme complexes perform best in lignocellulose hydrolysis when the same substrate is used for their production (Sukumaran et al. 2005).

The submerged fermentation (SmF) is the most widely used technology for the production of cellulases commercially. However, the problems of longer fermentation times and lesser productivity in the SmF demand the search for an alternative method. Solid-state fermentation (SSF) is a relatively economical fermentation

technology because of its lower capital investment and operating cost (Zhuang et al. 2007; Srivastava et al. 2015; Sajith et al. 2016). The economic analysis studies have also revealed that the unit cost of cellulase production in SSF is lower than that in the SmF method (Zhuang et al. 2007). Nevertheless, several limitations of SSF such as problems associated with the mass or heat transfer and monitoring of process parameters need be addressed by designing specific bioreactors (Mienda et al. 2011). Thus the development of SSF and its adoption as a preferred fermentation technology for future cellulase production at industrial levels can prove beneficial in lowering the enzyme production costs significantly. Furthermore, the conventional approach of optimization of fermentation conditions such as medium formulation (using low-cost components), pH, temperature, dissolved oxygen concentration, agitation speed, etc. is also effective in enhancing cellulase production (Acharaya and Chaudhry 2012; Saini et al. 2015a).

The recent studies in the field of biofuel production are also targeting the co-culture or consortium-mediated cellulase production (Saini et al. 2015a). The different members of a microbial co-culture may produce different components of the cellulase enzyme complex to form a complete system (Srivastava et al. 2015). Various studies have been reported showing successful improvement in cellulase production by employing different microbial co-cultures such as *Aspergillus niger* and *Trichoderma viride* from waste paper (Juwaied et al. 2010); *Aspergillus niger* and *Penicillium chrysogenum* from waste paper, cotton waste, and bagasse substrates (Jayant et al. 2011); *Aspergillus niger*; *Aspergillus oryzae* from sugarcane bagasse substrate (El-Deen et al. 2014); and so on. In nature, mixed populations of different microorganisms act synergistically to biodegrade the complex lignocellulosic biomass. Based on this idea, synthetic microbial consortia can be constructed and used for cellulase production as well as the direct hydrolysis of different biomass (Minty et al. 2013; Poszytek et al. 2016). However, the construction of such consortia needs elaborated studies because microbes are also known to show many negative interactions. Nonetheless, several studies have revealed enhanced production of glycosyl hydrolases (including cellulases) using artificial consortia, e.g., compost-derived thermophilic bacterial consortia capable of producing xylanases and endoglucanases in titres comparable to the commercial cocktails of fungal hydrolytic enzymes (Gladden et al. 2011) and the high cellulolytic activity in a consortium of 16 bacterial strains isolated from different habitats for biogas production (Poszytek et al. 2016).

8.7.3 Genetic Engineering for Improved Cellulases

Development of recombinant strains by genetic engineering is a popular technique for the enhanced synthesis or secretion of cellulases in the microbial strains. The genetic modifications can alter the proportion of synthesis of different cellulases in an organism, e.g., the introduction of an expression vector pAMH110 (containing promoter for EG1 endoglucanase and terminator for the strongly expressed main cellobiohydrolase 1, cbh1) in a *Trichoderma reesei* mutant strain produced a new

strain capable of overproduction of EG1 endoglucanase selectively (Harkki et al. 1991). *T. reesei*, though a hypercellulase producer, produces insufficient amounts of β-glucosidase in its complex, which can be increased by genetic manipulation of *T. reesei* genome. The overexpression of bgl1 gene in *T. reesei* RUT C30 under the control of a four-copy cbh1 promoter (containing repeated inserts of positive transcriptional elements and deficient in the potential glucose repressor-binding sites) resulted in an increase in the β-glucosidase activity, which showed a positive effect on the saccharification of corncob residues (Zhang et al. 2010). The inactivation of catabolite repressors by genetic modifications can enhance the synthesis of hydrolytic enzymes, e.g., disruption of creA gene, encoding for catabolite repressor protein, in *Acremonium cellulolyticus* resulted in enhanced production of cellulase and hemicellulase in the modified strain (Fujji et al. 2013). Trigenic recombination in *Penicillium oxalicum*, i.e., deletion of bgl2 (encoding for intracellular endoglucanase, when deleted increases expression of intracellular cellobiose having an inducing effect) and creA genes (gene for catabolite repressor) and overexpression the gene clrB (induces cellulase expression) constitutively under the promoter gpdA from *A. nidulans*, increased the filter paper activity by over 20-fold in the recombinant strain (Yao et al. 2015). The overexpression of nine β-glucosidase genes in *P. oxalicum* under the constitutive and inducible repressor caused 65-fold increase in the β-glucosidase activity, which led to the reduction in enzyme loads required for the saccharification of the delignified corncob residue (Yao et al. 2016). The literature shows many more reports on the successful engineering of different genes in various microbial strains resulting in recombinant strains with more efficient cellulolytic systems (Singh et al. 2017).

8.7.4 Protein Engineering for Improved Cellulases

Protein engineering is also an efficient technology for remodeling cellulolytic systems in the microbes for enhanced hydrolytic enzymes synthesis. Two major strategies for this, based on mutagenesis, include: (1) rational design and (2) directed evolution (Maki et al. 2009). Rational design requires detailed or partial knowledge of the protein structure, its active site, and the structure-function relationships. First the structure of the selected enzyme is elucidated using techniques such as X-ray crystallography. The target amino acid sequence is then altered by site-directed mutagenesis. The transformation and expression of modified genes generate a variety of mutants, which are characterized to identify the desired changes in the resultant proteins (Mohanram et al. 2013). Many rational design-based research studies have achieved success in targeted alteration in different cellulolytic enzymes and have improved their hydrolytic potential. The Cel5A endoglucanase (hyperthermostable) from *Thermotoga maritima*, when subjected to site-directed mutagenesis of amino acids around the active-site region and fusion of carbohydrate-binding module (CBM) with CBM1 from (CBHII) *Trichoderma reesei* and CBM6 from *Clostridium stercorarium* xylanase, resulted in a shift in optimal pH from 5 to 5.4, increased enzyme activity (10%) in the mutant (N147E); and engineered CMB

caused 14–18-fold increased hydrolytic activity toward Avicel (Mahadevan et al. 2008). The specific mutation in the catalytic site of cellulosome endoglucanase, Cel8A, from *Clostridium thermocellum*, increased the stability of the mutant enzyme at 85 °C temperature (Anbar et al. 2012). The site-directed manipulation of Cel12B gene, encoding for hyperthermostable β-1,4-endoglucanase in *Thermotoga maritima*, by inverse PCR (polymerase chain reaction) produced recombinant enzymes capable of exhibiting higher enzyme activities compared to the parent enzyme (Zhang et al. 2015).

Directed evolution or irrational design utilizes the DNA techniques such as error-prone PCR (epPCR) and DNA shuffling to randomly generate a library of gene variants, which are screened for the evolved proteins having desired traits (Maki et al. 2009; Mohanram et al. 2013). This approach, contrary to the rational design, is independent of the knowledge of the protein structure and enzyme-substrate interactions (Liu et al. 2010). The selection of high-performance mutants, however, is a challenge in this method. In the directed evolution, the larger the size of the library of variants, the greater the chances of getting mutants with desired properties (Maki et al. 2009). Reports, however, have shown that robust variants can be generated from different microbes, which are capable of producing more efficient hydrolases. The directed evolution of 5-glycoside hydrolase (Cel5A) from a cellulolytic bacterium, *Clostridium phytofermentans* ISDg (by error-prone PCR), generated mutant, which showed sustained thermostability as well as higher activity on the soluble substrates (Liu et al. 2010). Similarly, five variants generated during directed evolution of Cel5A in a different bacterium, *Thermoanaerobacter tengcongensis* MB4, by error-prone PCR, showed the expanded range of temperature for activity of their endoglucanases (Liang et al. 2011). More reports are available in support of the applicability of directed evolution in engineering enzyme with refined attributes.

8.7.5 Random Mutagenesis for Improved Cellulases

Random mutagenesis by chemical or physical mutagens is also an effective technique for strain improvement. Most of the fungal strains employed for commercial cellulase production have been improved by random mutation than the genetic modifications (Singh et al. 2017). For example, *T. reesei* RUT-C30, the popular fungal strain for commercial cellulase production, was produced from a QM6a strain after three rounds of random mutagenesis (Peterson and Nevalainen 2012; Singh et al. 2017). The random mutagenesis of *Aspergillus* sp. SU14 with repeated exposure to Co60 γ-rays, ultraviolet irradiation, and N-methyl-N′-nitro-N-nitrosoguanidine produced a mutant strain *Aspergillus* sp. SU14-M15 with higher cellulolytic potential, which is further improved manyfold by optimization studies under SSF conditions (Vu et al. 2011). Similarly, cellulase production in many microbial strains, such as Aspergillus *sp*., *Penicillium janthinellum* NCIM1171, *Trichoderma atroviride*, *Humicola insolens*, *Penicillium decumbens* 114, *Aspergillus terreus* AUMC 10138, *Fusarium oxysporum*, *Cellulomonas sp*., etc., has been improved substantially using the technique of random mutagenesis (Singh et al. 2017).

8.7.6 Recycling of Cellulases

Other techniques in reducing the cost of cellulases include the recycling or immobilization of cellulases. The cellulases can be recycled after completion of different steps of the cellulosic ethanol production process such as recycling after initial hydrolysis, recycling after fermentation, or recycling after distillation. Regardless of the step chosen, the efficient recycling of the enzymes using the liquid phase requires a desorption step from the solids (Lindedam et al. 2013). The enzyme immobilization includes physical adsorption to a solid phase, covalent coupling to soluble polymers, crosslinking with bifunctional reagents, entrapment in a gel phase, encapsulation, etc. (Garcia et al. 1989; Obeng et al. 2017). In case of cellulases, desorption of enzyme is a challenge in the physical adsorption method because these enzymes show strong bonding with the cellulosic materials (Gilbert and Tsao 1983; Lindedam et al. 2013). The natural affinity for the cellulose, however, enables cellulases to be recycled by re-adsorption onto the fresh substrate (Du et al. 2014). The studies have shown successful recycling of β-glucosidase entrapped in calcium alginate gel particles without significant loss in its activity for up to 20 rounds of recycling (Tsai and Meyer 2014); recycling of cellulases immobilized on nonporous and porous silica with retention of >50% activity for at least four rounds of recycling (Ikeda et al. 2015); and recycling of endocellulases immobilized on the activated carbon, zeolite, ion exchange resin, and polystyrene in two different shapes (drop or sheet) and mixtures thereof (Silva et al. 2017).

8.8 Conclusions

Second-generation biofuel production from the cellulosic biomass is considered among most promising technologies to meet future energy demands of the world. Commercialization of this technology at wide scale relies on the efficiency of the cellulose hydrolysis, which in turn is determined by the efficacy of the cellulases. The cost of the cellulases is a major bottleneck in the economic production of the cellulosic ethanol. The strategies, such as the exploration of potent cellulases from the culturable or non-culturable cellulolytic microbes, technological advancements in the genetic and protein engineering, mutagenesis, and enzyme recycling, hold huge prospects for improvement of cellulases. The cost reduction of the cellulases by employing these approaches can serve as an alternative to the economic infeasibility of the pursuit of cellulosic ethanol production technology extensively.

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9 Applications of Bacterial Polysaccharides with Special Reference to the Cosmetic Industry

Acharya Balkrishna, Veena Agarwal, Gaurav Kumar, and Ashish Kumar Gupta

Abstract

Bacteria from all taxa synthesize various valuable, structural, and functional polysaccharides. Bacterial polysaccharides are biodegradable, biocompatible, and naturally nontoxic biopolymers. The bacteria secrete polysaccharides into the environment. These polysaccharides are referred to as exopolysaccharides (EPS). These microbial polysaccharides are used in an extensive range of cosmetics, pharmaceutical, medical, agricultural, and food applications. Among these different drives, cosmetics are complicated polyphase systems. Main bacterial polysaccharides, which are xanthan gum and gellan gum, are regularly used as psychosensorial agents and viscosity controllers. Further bacterial polysaccharides, viz., bacterial cellulose (BC), hyaluronic acid (HA), and levan, contain biological properties such as skin regeneration and defense. These bacterial polysaccharides are essential active agents in cosmetic formulations. The nontoxic activities of these bacterial polysaccharides have been systematically assessed. Several studies have established the safety of cosmetic ingredients during their applications.

Keywords

Bacterial polysaccharide · EPS · Cosmetic formulations · Nontoxic · Biocompatible

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9.1 Introduction

Polysaccharides are composite carbohydrates; they are actually a combination of many sugars (Greek poly = many; sacchar = sugars). This carbohydrate is formed by chain arrangement of a number of monosaccharides (from 10 to more than 1000) (Van Soest et al. 1991). Major monosaccharides such as glucose, fructose, galactose, etc., represent an essential part of polysaccharides. Majorly three polysaccharides such as starch (retrieved from plant source), cellulose (retrieved from plant source), and glycogen (stored in human liver and muscles; a glucose form) are related to human nourishment. Polysaccharides have different classes based on their digestive properties, viz., digestible and nondigestible, but none of that is part of essential nutrients for human system (Siddiqui et al. 2017). Apart from their nutritional role, naturally occurring and/or artificially formed polysaccharides also have various roles in commercial food, dairy, confectionery, freeze-dried products, cosmetics, medicines, pharmaceuticals, and bioactive therapeutic applications (Ramalingam et al. 2014). Plants are so far the most common source, while animal foods and microbes are only a little source of polysaccharides. Among microbes bacteria have a vital role in producing commercial polysaccharides followed by fungus and others. Polysaccharides produced by bacteria are mainly designated as bacterial polysaccharides (BPS) and also have an important role in bacterial metabolism (Öner 2013).

9.2 Bacterial Polysaccharides (BPS)

A bacterium produces secondary metabolites which can be categorized into three categories: small-small molecule, big-small molecule, and non-small molecule. Non-small molecules cover a wider range of molecules of DNA to polysaccharides. Bacterial polysaccharides may classify as extracellular polysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (CPS), and teichoic acids (Pichersky and Gang 2000). EPS and CPS are associated with both gram-positive and gram-negative bacteria, while LPS and teichoic acids are part of gram-negative and gram-positive bacteria, respectively. These bacterial polysaccharides are part of bacterial cell wall and have structural complexity and diversity. BPS plays many roles in bacterial system such as survival of bacteria, colonization of surface, and complement-mediated phagocytosis which ultimately leads to poor antibody response of the host (Kenne and Lindberg 1983). Thus, these BPS in case of drug resistance would seem the principal agent for the development of remedial intervention. BPS also contributes in the interaction between bacteria and plant systems. Till date there are a number of EPS that were characterized at the molecular level, but only a few were established for industrial applications. Industrial applications and/or full commercialization of BPS were slow due to the cost of production (mainly substrate cost) and expensive overall purification procedures (fermentation process, downstream processing). However, substrate cost may be overcome by using cheaper substrate, while optimizing of the whole purification

process makes a cost-effective production of the BPS (Sutherland 1998). Similarly, cost-effectiveness of production of BPS can be enhanced by using high-yielding strains. Among the number of polysaccharides, some are an alternate source of algae (e.g., carrageenan) producing polysaccharides and plant-producing polysaccharides (e.g., pectin) which have traditional applications. These traditional replacing polysaccharides, namely, are bacterial cellulose, levan, etc. Other extensive applications of BPS are nutraceuticals, functional food, cosmetics, pharmaceuticals, insecticides, and herbicides. In the current scenario, BPS can also be used as anticoagulant, immunomodulator, anticancer, and other biological agents (Lapasin and Pricl 1995).

Most of the polysaccharides are found to be in positive correlation with pathogenic state. These polysaccharides are referred to as exopolysaccharides, and they may either be homopolymeric or heteropolymeric in composition and of diverse high molecular weights (10–1000 kDa). In comparing to traditional polymers, BPS have more functional properties. This characteristic importance of BPS encourages developing more bacterial polysaccharides and releasing them to the market. Moreover, chemical and/or traditional polymers fail to act in accordance with standard regulations in the degree of purity with less and/or specific functional importance. BPS also have unique physicochemical properties (such as pH, ash content, moisture, solubility, etc.) and high-value applications (Nwodo et al. 2012). Commercial production of BPS has a real less cost of production with higher yield. This article reviews the application of BPS in biotechnological area with special reference to cosmetics. It also lists the important BPS names and structures and their producer bacterial genus.

9.3 Biotechnological Importance of BPS

There are already a number of bacterial polysaccharides that have been discovered and documented (Nwodo et al. 2012). In the current chapter, we are trying to put all recent updates of the biotechnological importance of bacterial polysaccharides. The biotechnological importance of any substance mainly involves the advancements in applications. Here, the biotechnological importance of these polysaccharides has been categorized mainly in four sectors, viz., food, health, and agricultural and industrial sectors (Fig. 9.1).

9.3.1 Food

Bacterial polysaccharides have wide applications in food industries from food additives to probiotics. There are a number of polysaccharides which had been approved by the FDA and now have a major role in food processing industries. Xanthan gum which is secreted by *Xanthomonas campestris* is being used in food such as salad dressing, frozen foods, beverages, and sauces. Xanthan gum, used at concentrations, ranges from 0.01% to 0.05% as per the requirement. Gellan gum

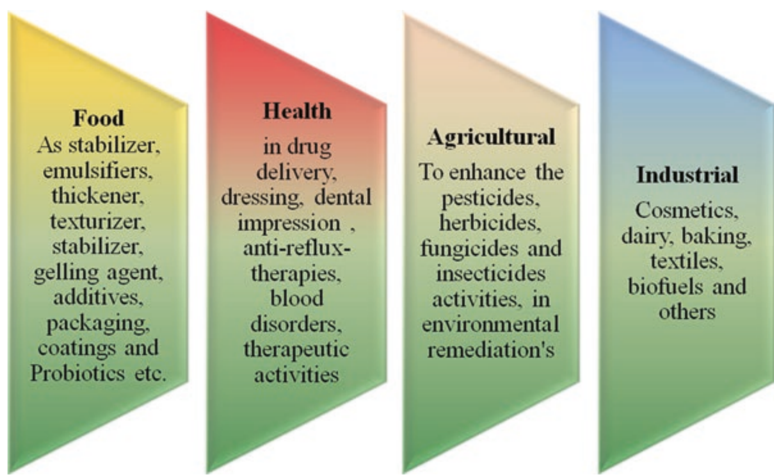

Fig. 9.1 Illustrating the biotechnological importance of BPS

has been used as a gelling agent and texturizer (concentration ranges from 0.05% to 0.40%). Pullulan polysaccharide provides pH stability of food, while dextran has application in frozen foods. Xylene, alginates, and curdlan polysaccharides were used as gelling agents for food. Currently, BPS as functional food product is becoming an important member of the food supply chain which benefits not only human health but also fulfills the nutritional requirements. A number of studies have proven that disease prevention can be done by diet and its components. However, detailed studies are required in bacteria producing EPS and their impact on human health and nutritional supply. BPS from lactic acid-producing bacteria, especially from *Lactobacillus* species, could be the major source of food polysaccharides (Ramalingam et al. 2014).

9.3.2 Health

The health importance of any substances covers the pharmaceutical to therapeutic applications. Bacterial polysaccharide applications in health sectors can be seen as in drug delivery systems, in wound dressing, and in oral care. BPS such as dextran and sulfated forms of alginates has been reported to prevent blood disorders. So, these forms of BPS could be an alternative of natural heparin and plasma deficiency. Other therapeutic activities of BPS include anticoagulant, antimetastatic, anti-angiogenesis, anti-inflammatory, and antithrombotic actions. Similarly polysaccharides from marine source were also reported to be used in cell therapy and in regenerative medicine. Bacterial polysaccharides from *Vibrio* spp. can be used in the treatment of spinal cord injury as well as in bone integrity (Roberts 1995).

9.3.3 Agricultural

Bacterial polysaccharides have better suspending nature and compatibility with salt which makes them a useful candidate in agricultural as well as in farming practices. Bacterial polysaccharides have a major role in biocontrol bacterial cells (e.g., *Bacillus subtilis* exopolysaccharides) to interact and/or control the plant fungal pathogens (*Fusarium oxysporum*). Bacterial polysaccharides also enhance the pesticide, herbicide, fungicide, and insecticide activities. They are also having some major role in environmental bacterial metabolism which helps in detoxification of toxic forms of metal to nontoxic forms (hexavalent chromium detoxification in to nontoxic form by *Bacillus* spp.) by bacterial cells. BPS also has a major role in developing water-based lubricants which have the main role in water conservation area (Morris 1991).

9.3.4 Industrial

Bacterial polysaccharide application in the industrial sector includes cosmetics, dairy, baking, textiles, biofuel industries, etc. Cosmetic applications of BPS and their mode of action in brief will be discussed below. Application of BPS in dairy industries includes stabilization of dairy products as well as viscosity control of mixed products. In baking industries BPS acts as stabilizer agent and decreases the bread staling capacity of baked products. It also has a role in optimizing different parameters of baked products such as water retention, moisture, texture, and bread volume. In textile industries, bacteria-based polymers are used in the production of water-resistant cloths such as raincoats and vehicle covers. In biofuel production BPS enhances the oil recovery fluids and has some role in oil field drilling (Singh et al. 2008).

9.4 Cosmetic Applications of BPS

Bacterial polysaccharides may be used in the form of medicines (mentioned above) and in cosmetics. The current era of human society also raised the application of natural sources in personal care. In personal care products such as cosmetics, it plays a vital role in enhancing human body appearance. These cosmetics can be categorized as sunscreens, shampoos, conditioners, lotions, creams, and dyes. Cosmetics make contact with the epidermis, hair system, oral cavity, and nails. Cosmetics do not affect the body functions and structure, and there is no premarket approval required. However, their active ingredients and concentrations must be regulated. Cosmetics have multiphase systems which include base, active, and additive substances. Microbial source of polysaccharides is an important source of

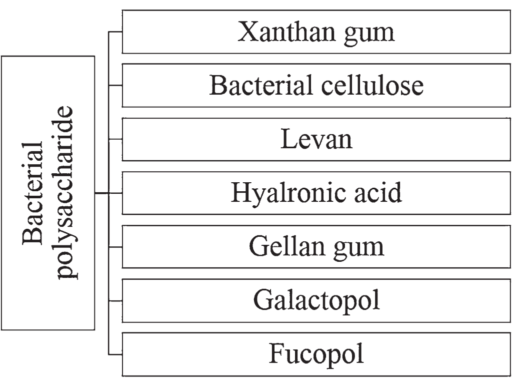

Fig. 9.2 Major bacterial polysaccharides of cosmetic industries

a commercial market. In cosmetics, major BPS are xanthan gum, bacterial cellulose, levan, hyaluronic acid, gellan gum (Fig. 9.2; Table 9.1), and two newly discovered polysaccharides galactopol and fucopol (Table 9.1).

9.4.1 Bioactive Bacterial Polysaccharides of Cosmetic Industries

Bacterial polysaccharides are very useful and have multiple applications in the cosmetic industry as they can be used as film formers, viscosity-increasing agent, binder emulsion stabilizer, and skin-conditioning agents (Fig. 9.3).

9.4.1.1 Xanthan Gum

It is a heteropolysaccharide with glucose backbone (Fig. 9.4). This glucose backbone is linked through beta glycoside linkages with trisaccharide side chains on each alternate glucose residue. It is a major and most widely accepted commercial microbial polysaccharide for food and cosmetics. It can solubilize in water and has shear-thinning behavior. It is synthesized by *Xanthomonas campestris* in larger quantity. It is produced and marketed by major pharmaceuticals and cosmetic firms. It is applicable in cosmetics as oral hygiene products, deodorants, baby products, etc. Xanthan gum acts as a binder, skin-conditioning agent, emulsion stabilizer, and surfactant at 4–6% concentrations in hair dye and nail products. Lower concentrations (0.05–0.5%) are used in deodorants, baby products, and spray (Imeson 2010).

9.4.1.2 Bacterial Cellulose (BC)

It is a high-molecular-weight, glucose homopolysaccharide (Fig. 9.4). It is secreted by *Gluconacetobacter xylinus, G. hansenii, G. pasteurianus*, *Agrobacterium*, *Alcaligenes*, *Rhizobium*, *Pseudomonas*, etc. (Mohite et al. 2013; Barnhart et al. 2014). Bacterial cellulose can be produced by using sugar as carbon source, but at small scale. It cannot be produced in large scale due to the long culture period. Because of these limitations, BC is mostly restricted to high-value products. Because of its skin regeneration property BC may be useful in skin repairing, wound healing, and moisturizing the skin. BC is useful in formation of base of artificial nail and

Table 9.1 Major BPS of cosmetic applications

Bacterial polysaccharides (BPS)	Charges	Molecular weight	Producer	Major ingredients	Applications	References
Hyaluronic acid	Anionic	2.0×10^6	*Streptococcus* sp.	Acetylglucosamine, glucuronic acid	Antiaging affect Lubricant, Immunostimulant, Angiogenic	Vázquez et al. (2010, 2013), Liu et al. (2011) and Berkó et al. (2013)
Gellan gum	Anionic	5.0×10^5	*Sphingomonas paucimobilis*	Glucose, acetyl, rhamnose, glucuronic acid	Filmogenic Gelling agent	Fialho et al. (2008), Ullrich (2009) and Imeson (2010)
Xanthan gum	Anionic	$2.0–5.0 \times 10^6$	*Xanthomonas* sp.	Mannose, glucose, glucuronic acid	Hydrocolloid	Rottava et al. (2009), Ullrich (2009) and Imeson (2010)
Levan	Neutral	3.0×10^6	*Bacillus subtilis*, *Zymomonas mobilis*	Fructose	Anti-inflammatory, skin irritation alleviation, skin moisturizing, skin whitening	Oliveira et al. (2007) and Kang et al. (2009)
Bacterial cellulose	Neutral	1.0×10^6	*Gluconacetobacter xylinus*	Glucose	Skin regeneration, skin moisturizer, wound healing	Chang-Chun (2009), Amnuaikit et al. (2011) and Fu et al. (2013)
Glucopol	Anionic	$1.0–5.0 \times 10^6$	*Pseudomonas oleovorans*	Galactose, mannose, glucose, pyruvate	Skin moisturizing Sun protection	Freitas et al. (2009, 2011)
Fucopol	Anionic	$2.0–10.0 \times 10^6$	*Enterobacter A47*	Fucose, galactose, glucose, pyruvate	Skin moisturizing Sun protection	Freitas et al. (2009, 2011)

preparation of facial mask. It is also used in the formation of natural scrubs, aloe vera extract, olive oil, and ascorbic acid (Hasan et al. 2012).

9.4.1.3 Levan

It is a homopolysaccharide which contains fructose residues joined by beta linkages. It is present in plants, fungi, and bacteria. It arises via microbial transformation from sucrose carried out by the action of an enzyme levansucrase. Levansucrase is produced extracellularly by some bacterial genus species, viz., *Pseudomonas, Erwinia, Streptococcus*, and *Zymomonas* (Table 9.1) (Senthilkumar and Gunasekaran 2005). It is widely applied in cosmetics, pharmaceuticals, and food industries. It does not have water swelling capacity and has very low intrinsic viscosity value (0.14 dl/g). Its biological activities such as cell proliferation and skin repairing and moisturizing make it an important part of a three-dimensional artificial skin model. It protects cells, protective against the irritation agents. Levan BPS decreases the water loss from skin and keeps skin moisturized (Kang et al. 2009). Despite its many biological properties, levan applications in cosmetics are still limited because of its weak stability in aqueous formulations. Under the acidic conditions, levan hydrolyzes into fructose.

9.4.1.4 Hyaluronic Acid (HA)

It is a high-molecular-weight glycosaminoglycan component. It is a key structural component of the mammalian connective tissue and helps in controlling tissue permeation and hydration, bacterial invasiveness, and macromolecular transport between cells. It has wide physical and biological property due to its high swelling capacity. Properties such as biocompatibility, viscoelasticity, lubricity, and angiogenic and immunostimulatory effects are enriched with HA (Allemann and Baumann 2008; Vazquez et al. 2013). Activity of HA is fully dependent on molecular size and weight. Its application in medicine and cosmetics, increases its market value.

It is one of the most valuable BPS used in cosmetics, medicines and pharmaceuticals due to its biocompatibility, biodegradation, viscoelasticity.

Earlier HA was obtained from mammalian tissues, but due to public health concern at the time, it became absent in the market. Microorganisms and marine algae are the source for HA. Examples of these microorganisms are pathogenic streptococci (e.g., *Streptococcus equi* subsp. *zooepidemicus*), including wild-type and attenuated HA-high-producing mutants. Due to the pathogenic development of recombinant strains, nonpathogenic microorganisms (especially generally recognized as safe (GRAS) strains for the production of HA) make an alternative for pathogenic bacterial strains (Kim et al. 1996). These nonpathogenic strains are recognized as GRAS strains. Transformation is an approach for HA production in nonpathogenic organisms. However, restrictions for use of recombinant strains in a number of countries and their production may be achieved by heterogonous HA-producer system.

Substrate cost is not a major concern due to the high market value of this BPS. The important factors are strain, process development, and quality of HA

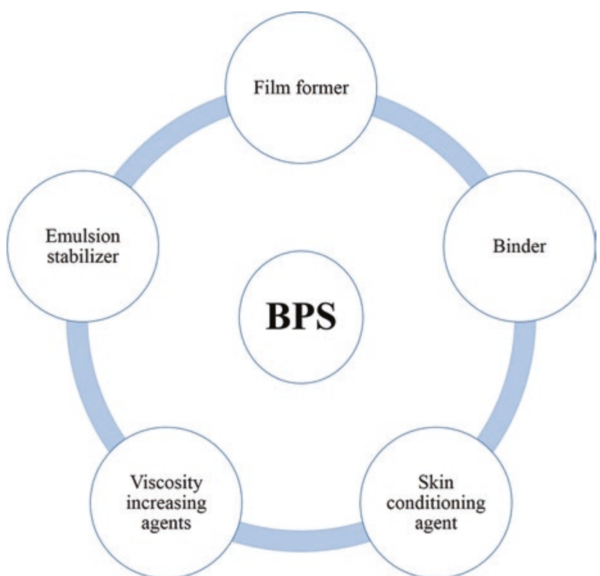

Fig. 9.3 Applications of bacterial polysaccharides in cosmetics

(Chong et al. 2005). Bacteria which are responsible for HA production require a complex growth media containing glucose, nucleotides, amino acids, vitamins, and trace elements. There are some articles which report agro-industrial wastes/by-products as a carbon source for the production of HA. Similarly whey protein concentrate, soy protein concentrate, cashew apple juice, corn steep liquor, wastewater, and peptone from fish source are also confirmed as a carbon source for HA-producing bacteria. HA from natural sources (bacterial fermentation) may be used in skin cream, in conditioners, and in other cosmetics. HA skin hydration and water retention capacity makes it an important ingredient of cosmetic antiaging creams and anti-wrinkle cream. HA and its salts are used in various categories, viz., makeup lotions, moisturizers, and lipsticks. HA concentration of 1% enhance moisture and skin elasticity (Allemann and Baumann 2008).

The HA may be used as dermal filter and can replace collagen in nonsurgical cosmetic procedures. These fillers are having differences in cross-linking agent, percentage of cross-linked HA, elastic modulus, etc. These filters do not require any prior skin allergy testing and have long clinical effects.

9.4.1.5 Gellan Gum

It is a heteropolysaccharide with a tetrasaccharide backbone with glucose, glucose monomers, glucuronic acid, and rhamnose. Its side chains are made up of acetyl and glyceryl substituents (Coleman et al. 2008). *Sphingomonas paucimobilis* (accession no. ATCC 31461) is the bacterial strain used for industrial production of gellan gum (Bajaj et al. 2007). As a carbon source these bacteria use simple sugars, which are the most common carbon sources. It forms gels under cooling and has gel properties. Gel properties are determined by gellan's acyl groups content. High acyl content produces soft, elastic, thermo-reversible, non-brittle gels, whereas firm, nonelastic,

Fig. 9.4 Structure of major bacterial polysaccharides (**a**) xanthan gum, (**b**) gellan gum, (**c**) levan, (**d**) hyaluronic acid, and (**e**) bacterial cellulose

brittle, and thermostable gels are produced by low acyl forms. The properties of low acyl gellan gel are dependent on the presence of cations and pH, while high acyl gellan gels are not as sensitive to the pH ionic environment. It is used in cosmetic formulations majorly as a viscosity increase agent and an emulsion stabilizer. It is regularly used at lower concentrations of 0.3–0.5% in dermal products, while in other products such as hair products, powders, and eye products, used in concentrations of 0.0004% (Fiume et al. 2016).

9.4.1.6 Galactopol

Galactopol is a newly reported BPS, and it has major components such as galactose, mannose, glucose, rhamnose, acetate, and pyruvate. Galactopol has anionic charges with the molecular weight of $1–5 \times 10^6$ (Table 9.1). Galactopol are biosynthesized by a bacterium (*P. oleovorans*) growing on glycerol. This low-cost substrate is a by-product of biodiesel industries and acts as carbon source for growing bacterial culture. It has viscous shear-thinning nature in aqueous solution and medium. It also acts as an emulsifying and flocculating agent and is used in skin moisturizers and lotions (Freitas et al. 2009, 2011).

9.4.1.7 Fucopol

Fucopol has fucose, galactose, glucose, acetate, and pyruvate. It has anionic charges with the molecular weight of $2–10 \times 10$ (Berkó et al. 2013). Fucopol biosynthesized likewise galactopol and also uses glycerol as a carbon source. Fucopol production is bacterial growth-associated EPS production, which means the major quantity of this BPS is produced by bacteria in log phase of growth cycle. It is majorly produced

by *Enterobacter* A47 bacterial strains and has galactopol-like properties and applications in the cosmetic sector (Table 9.1). Since fucopol is the main source of fucose, it has various bioactive roles such as antitumor and anti-inflammatory (Freitas et al. 2009, 2011).

9.4.2 Cosmetics BPS: Mode of Action on Human Skin

Human skin is made up of three layers, viz., the epidermis, the dermis, and the hypodermis. Epidermis which is the outermost layer consists of several cell layers which are being regenerated from the basal cell layer in the stratum corneum. The dermis layer is composed of collagen and glycosaminoglycans. This layer contains blood vessels, glands, nerves, and hair follicles. The hypodermis or subcutaneous layer consists of adipose tissue which acts as a thermal barrier. Stratum corneum is a highly lipophilic layer which protects the inner layers from environmental stimuli. It has 13% water, while the inner layers are more hydrophilic (water content of viable epidermis is 50% and of dermis is >70%). Degree of hydration affects the skin permeability. Skin barrier functions are influenced by age and external factors as well as by body locations.

Loss of water content of the skin causes dryness, roughness, cracks, and itching. Dry skin epidermis has less moisturizer retention ability which can be treated by enhancing hydration and reducing water evaporation. Tropical moisturizer can also be used for this treatment. Transport of molecules occurs across the skin via lipid bilayers between cells and directly through the cells or gland ducts or hair follicles. It can transport only low-molecular-weight (< 500 Da) lipophilic molecule with bi-solubility (in lipophilic stratum corneum and the hydrophilic inner epidermis layer). Skin permeation can be enhanced by only disruption of structural organization of stratum corneum. Disruption can be occured by physical techniques (e.g., electroporation, microneedling) or chemical enhancers (e.g., biodegradable polymers) or (Ammala 2013). Skin exposed to the harmful environment, such as UV radiation, can lead to mutation, premature skin aging, or skin cancer. UV radiation causes peroxidation of the lipid matrix of the stratum corneum and leads to loss of function of the structure's barrier (Trommer and Neubert 2005).

9.4.3 Toxicity Essentials

Safety of cosmetic products relies on the safety of its active ingredients which may be evaluated by a series of toxicological testing. This series of testing determines the toxic potential of any cosmetic ingredient (Van Essche 2001; Chew and Maibach 2001). These are the following tests which are required to set the base:

(i) Acute toxicity
(ii) Corrosivity and irritation

(iii) Skin sensitization
(iv) Pre-cutaneous/dermal adsorption
(v) Repeated dose toxicity
(vi) Genotoxicity/mutagenicity
(vii) Further tests includes reproductive toxicity, carcinogenicity

9.5 Conclusions

Bacterial polysaccharides have physicochemical and/or biological properties which make them key ingredients in biotechnological areas and in cosmetic products. However, a limited number of biopolymers are currently in application as cosmetic ingredients. Xanthan gum is the most relevant in cosmetics due to its viscosity-increasing capacity at low concentrations. Other BPS such as bacterial cellulose, hyaluronic acid, and levan are also used as bioactive ingredients, while gellan gum is used in producing hydrogels and/or rheology modifiers. Apart from these applications, hyaluronic acid is utilized in cosmetic vehicles such as nano-emulsions and hydrogels. As the launching of new cosmetic product does not require any extensive regulatory requirements for marketing, the door opens for application of BPS as cosmetic ingredients. However, their toxicity and safety must be recognized.

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Polyphenol Oxidase, Beyond Enzyme Browning

10

E. Selvarajan, R. Veena, and N. Manoj Kumar

Abstract

Enzyme browning is a usual phenomenon that can be observed commonly in fruits and vegetables, which results in quality loss of the food including the change in color, taste, flavor, and nutritional value. This occurs when the phenolic compounds present in them react with polyphenol oxidase (type III copper enzyme). The phenolic compounds are oxidized to their quinone derivatives and further oxidized to form melanin pigment, found in living beings, which is responsible for the browning reaction. Methods to prevent browning, during the process and storage, are the matter of immense concern in the field of the food and food processing industry. While we are discussing about the disadvantage in the food industry, it contributes significant usage in the drugs, water/soil treatment (bioremediation), and textile industries. Thus, this article elucidates the science behind enzyme browning: its significance, cause, effect, remedy, and efficient applications in different industrial sectors.

Keywords

Polyphenol oxidase · Genetic significance · Production · Immobilization · Diversity

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10.1 Introduction

Polyphenol oxidases (PPO) or tyrosinases or monophenol monooxygenases are enzymes having dinuclear copper center with aromatic rings that allows the molecular oxygen to cleave at the ortho-hydroxyl group followed by the subsequent hydroxylation of monophenols to o-diphenol (catechol oxidase) and oxidation of o-diphenol to quinone. The PPO product formed is also responsible for melanogenesis that results in pigmentation in animals (López-Molina et al. 2003). During this reaction, the melanin-like insoluble components can form in the aqueous substrates as tyrosinase converts both DOPA and tyrosine to melanin and catechol oxidase converts only DOPA to melanin (Hammer 2013). To ensure the production of o-diphenols, the reducing agents like ascorbic acid or bi-sulfites (Ricquebourg et al. 1996) can be added (Doddema 1988). The first step is reversible. The o-quinone formed undergoes nonenzymatic polymerization reaction with the other quinones, amino acid, and proteins to form the dark red, brown, and black compounds. These dark compounds are observed in the damaged fruits and vegetables and are reported to lower the nutritional value (Ni Eidhin et al. 2010). This enzymatic browning occurring causes undesirable changes in the quality of the food during processing and storage. The browning activity is found to be directly proportional to the amount of phenolics present in the plants (Pilar Cano et al. 1997). Thus the food industries prefer less PPO activity in the fruits and vegetable for higher shelf life. There are researches going on to control the production of PPO in natural foods. The freshly cut fruits/vegetables are more susceptible to browning activity, but this enzyme is preferred in places where the color is desirable like tea, coffee, and cocoa (Toledo and Carolina 2016). These PPOs or metalloxides are also observed in prokaryotes that have activity on certain metals like Cu (I) and Fe(II) and are applied in metabolism of metal (Martins et al. 2015).

The polyphenol oxidase is coded by a gene family having multiple genes that are highly conserved. The active center of the enzyme has six to seven histidine residues attached to each copper atom while present along with the highly conserved cysteine residue. The two copper centers in PPO, namely, Cu-A and Cu-B, are found to have a 42% similarity between bacteria and humans (Jukanti et al. 2004). In higher plants, they are found in the thylakoid lumen and chloroplast membrane, while their phenol substrates are found in vacuoles (*Antirrhinum majus* L. (snapdragon) (Tran et al. 2012), giving us a hint to correlate them with photosynthesis (Boeckx et al. 2015). It is said that the PPO will not be active when it is not inside the plastid. But evidences have proved that the PPO also coexists in cytoplasm in the degenerating or senescent tissues (Hammer 2013). It also contains N-terminal peptides for the plastid transport. These peptides play a defense role near the seed surface (Palma-Orozco et al. 2011). The tropolone, being a very good copper chelator, is said to inhibit the tyrosinase activity while serving as a substrate for peroxidase. The oxidation of tropolone is done only if both the peroxidase and hydrogen peroxide are present (Hammer 2013). PPO activity has been observed in different fruits and vegetables such as peach (Flurkey and Jen 1980),yali pears (Cheng et al. 2015), mushroom (Burton et al. 1993), walnut leaves (Zekiri et al. 2014), broccoli florets (Gawlik-Dziki et al. 2007), embryogenic and non-embryogenic cotton (Kouakou

et al. 2009), grapes (Lamikandra et al. 1992; Wissemann and Lee 1985), taro (Duangmal et al. 1999), banana (Yang et al. 2000), tomato (Oller et al. 2005), loquat (*Eriobotrya japonica* Lindl.) (Martínez-Márquez 2013), wheat kernels (Jukanti et al. 2004), apple (Ni Eidhin et al. 2006), and mamey (Palma-Orozco et al. 2011) and mainly in many different parts of plant like root plastids, potato tuber amyloplast, hypocotyl plastids, epidermal plastids, carrot tissue culture plastids, leucoplasts in *Aegopodium*, apical plastids, and etioplasts, chromoplasts, and chloroplasts of many species (Hammer 2013). Thus recent studies have shed some light on the functions of PPO in plants. Apart from plants, it is also present in fungi, bacteria, and animals but more research should be done to explore the biological functions of PPO in plants and fungi.

Few companies like Novo Nordisk (US5356437), Norozymes, Novozymes (US6242232), the Procter and Gamble company (WO1991005839 A1), and Albert Kaltenegger (WO1993023600 A1) have patented for application of PPO in removal of excess dye from new textiles, bacterial PPO from *Bacillus* for use in oxidation of colored substances, dye transfer inhibition, enzymatic preservation of water based paints, process and device for treating textiles etc. But in general, the global industrial enzymes market is predicted to reach USD 6.30 Billion by the year 2022 in terms of value, at a CAGR (Compound Annual Growth Rate) of 5.8% from 2017. Similarly, the food antioxidants market obtained a profit of USD 1.05 Billion in the year 2015. It is predicted to increase at a CAGR (Compound Annual Growth Rate) of 5.10% from the current year to reach 1.48 Billion by 2022. Thus like any other enzymes, the PPO too has great industrial applications in the waste water treatment and textile industries.

10.2 Insight into Polyphenol Oxidase

Polyphenol oxidases (PPOs) are a group of proteins with a copper center, which is found to be distributed from single-celled to complex organisms in the phylogeny. It is said to oxidize o-phenols and convert them into o-quinones which in turn produces stygian pigments on the layer of damaged tissues. These metalloproteins which have copper active site are capable of transporting and participating in O_2 metabolism. Based on the spectroscopic studies, the Cu proteins are classified into four major families (Toledo and Carolina 2016):

Type I: It has a blue active center which has copper atom and is responsible for transfer of electrons – a key concept of redox reactions.

Type II: They lack a copper at its active center but exhibits galactose oxidase activities. This includes dopamine-β-monooxygenase.

Type III: These enzymes have their active site with two copper atoms. They are involved in the oxidation process and transportation of the available O_2.

Type IV: They are recently identified in the *Streptomyces murayamaensis* and are reported to produce hydrolytic enzymes that convert o-aminophenol to o-iminoquinone. In contrast with the other enzymes that have tyrosinase activity, they do not hydroxylate monophenols.

The PPO falls under the class III enzymes (Toledo and Carolina 2016). The PPO has been found in the process of pigment formation and other defense mechanisms against plant pathogens and herbivory insects where they scavenge oxygen. Physical polyphenol barriers are built by the phenolic compounds that limit the pathogen movement into them. For example, the quinones that are formed as a by-product can form complex with plant proteins and reduce the digestibility of that protein in herbivores. They are also said to reduce the nutritive value. Meanwhile, the phenolic substrates which are oxidized by PPO are said to be one of the main reasons behind the browning in most of the fruits and vegetables throughout storage and processing. Thus a major problem faced by the food industry is when this affects the nutritional quality and appearance of the food. It also reduces the consumer's willingness to buy them thus reducing the total economy of the food producer and processing industry. It is approximated that over a 50% of loss in food industry occurs due to browning. Tropical fruits and vegetables are very likely to be prone to enzyme browning. PPO has been considered to play a crucial role in food technology, and a detailed study is done in several plants. As described earlier, the plant PPO are initially synthesized as pre-proteins that carries putative plastid transit peptides at the N-terminal region, which helps to target the enzyme into the thylakoid (Toledo and Carolina 2016).

10.3 Science Behind Enzyme Browning

PPO plays a major part in the development of brown pigments in plant. The major step involve in the browning reaction is conversion of monophenols or diphenols into quinone due to the synthesis of PPO when the tissue get damaged. One of the most difficult tasks is to determine if PPO is directly involved in browning or if the reaction is due to the result of any other metabolic event. There are some cases that confirm the browning reaction is mainly due to the secondary metabolic events. This is demonstrated in a work where the blackheart development in pineapple was observed after freezing it (Zhou et al. 2003). PPO, which earlier was found to be involved in the browning reaction, is now found to have another role in the development of blackheart. Similar development of blackheart is also found in pears due to PPO activity, but the level of PPO and the phenolic substrates doesn't seem to restrict the formation of the brown-core disorder (Veltman et al. 1999).

10.4 Phenol and Its Functions

Phenols and its derivative compounds are secondary plant metabolites and show no prominent role in the developmental stages of plant and are synthesized by shikimic acid pathway and later produced using carbohydrate metabolic intermediate compounds (Barberán et al. 2001).

In general, phenol is depicted as XOH, where X is supposed to be an aryl or phenyl compound, i.e., naphthyl. Polyphenols basically are the derivatives of the

phenol family. Methylphenol is a unique name given to cresols and is comparatively larger in size like the condensed tannins. All these varieties of phenolics play similar roles in food industry, which include oleuropein, benzoic acid derivatives, lignans, stilbenes, anthocyanins, cinnamates, isoflavones, flavanones, flavones, flavonols, proanthocyanidins, chalcones and dihydrochalcones, tannin-like compounds, ellagitannins, and other phenols. These phenolics have distinct chemical structures and a variety of biological functions. Among them is that they are found to have antioxidant, anticancer, anti-inflammatory, and estrogenic activities, suggesting their usage in coronary heart disease and tumor prevention. They also play a noticeable part in determining the appearance of food. For example, the anthocyanin pigments are accountable for the color formation like red or the hue shades obtained in plant-derived food but may look black in few of the commodities. Phenolics may also change the flavor of certain foods to sweet, bitter, pungent, or astringent taste and may also be a key factor to aroma in certain foods like coffee (Mazzafera and Robinson 2000).

As listed in the Table 10.1, tyrosine and chlorogenic acids are the most common substances which arise by nature and could possibly be the substrate in any plant-based food (Waleed et al. 2009). Although the qualitative and quantitative dependency of phenolic content of plants depend basically on their genetic build, the natural parameters like irrigation facility, soil content, nutrient availability, and the process and storage conditions also influence the phenol content in the plants (Barberán et al. 2001).

Phenolic compounds are found in industrial wastes obtained from coal and lignite plants, oil and petroleum refineries, paper industry, etc. The compounds obtained are toxic to plants, animals, and humans. It also imparts an unpleasant odor and taste to the drinking water, even when present at an extremely less concentration (Russell and Burton 1999).

10.5 Mélange of Phenol Oxidative Enzymes

In nature, there are numerous classes of enzymes that oxidize phenolic compounds. They are broadly classified into two major categories:

1. Peroxidase
2. PPO

10.5.1 Peroxidase (PPD)

Peroxidases are metalloproteins that contain the “heme” prosthetic group in them and are predominantly produced by various plants and microorganisms. Later, these enzymes catalyze further reactions by using hydrogen peroxide (Durán and Esposito 2000). These enzymes are further divided into different subclasses.

Table 10.1 Major phenols present in plants

Source	Substrate	pH	Temperature	Reference
		Optimum	°C	
Mushroom	Catechol, tyrosinase	7	20 °C	Öz et al. (2013)
Sweet potato	Chlorogenic acid, 4-methylcatechol	7	30 °C	Manohan and Wai (2012)
Potato	Tyrosine, chlorogenic acid, flavonol	6.4	22 °C	Waleed et al. (2009)
Apple	Chlorogenic acid (flesh), catechins (peel)	7	20 °C	Waleed et al. (2009)
Eggplant	Caffeic, coumaric, cinnamic acid derivatives	7	22 °C	Waleed et al. (2009)
Coffee bean	Chlorogenic and caffeic acids	6–7	30 °C	Mazzafera and Robinson (2000)
Butter lettuce	Chlorogenic acid, epicatechin	4.5 and 7.8	30 °C	Fujita et al. (1991)
Broccoli	Catechol, 4-methylcatechol	5.72	25–30 °C	U. G. Dziki et al. (2007)
Grapes	Dopamine/catechol	5.0–5.6	25 °C	Fronk and Jaeckels (2016)
Blueberries	4-Methylcatechol, DL-DOPA, catechol and dopamine	6.1–6.3	35 °C	Siddiq and Dolan (2017)
Avocado	L-DOPA or catechol	5–7	25 °C–60 °C	Toledo and Carolina (2016)
Mango kernel	Catechol	6	25 °C	Arogba et al. (1998)

10.5.1.1 Horseradish Peroxidase (HRP)

HRP is a subtype of peroxidase that is capable of catalyzing the oxidation reaction in phenols, biphenols, anilines, benzidines, and other concomitant hetero-aromatic compounds. Horseradish peroxidase perpetuates its activity under different optimal conditions and hence is ideally said to be pertinent for treating wastewater (Durán and Esposito 2000).

10.5.1.2 Chloroperoxidase (CPO)

Caldariomyces fumago, a fungal strain, is promulgated to produce the CPO enzyme which is said to oxidize the phenols. It is also said to oxidize chloride ions and ethanol to aldehyde compounds (Durán and Esposito 2000).

10.5.1.3 Lignin Peroxidase (LIP)

LIPs are again a subclass of peroxidase enzyme that is reported to be produced from the ascomycetes and basidiomycetes such as *Phanaerochaete chrysosporium*. Lignin peroxidase acquired from various origins is examined and was observed that it mineralizes a wide range of recalcitrant aromatic compounds and oxidizes a variety of cyclic phenols (Durán and Esposito 2000).

10.5.1.4 Manganese Peroxidase (MnP)

Manganese peroxidase is said to initiate the oxidation reaction in many mono-aromatic phenols and aromatic dyes, depending on the manganese and buffer used. It is said to act on phenolic compounds and dyes, in its free form (Durán and Esposito 2000). The enzyme requirement for the wastewater treatment and related applications is very less thus making it practical for the usage.

10.5.2 Polyphenol Oxidase (PPO)

PPOs are oxidoreductase enzymes that are said to catalyze the oxidation reaction in phenols and are further divided into two subclasses, i.e., tyrosinase and laccase. Both of these classes are found to react with atmospheric O_2 without any cofactors (Durán and Esposito 2000) and initiate compound transformations (Durán et al. 2002).

10.5.2.1 Tyrosinase

Tyrosinase, which has been identified with many other names like monophenol monooxygenase, o-diphenol oxidoreductase, and catechol oxidase, is found to have a wide distribution throughout the phylogenetic scale from single-celled to complex multicellular organisms. It is also found to be present in many isoforms in different tissues of the same organism, like root and leaves of the same plant species. Previous studies report that the tyrosinase enzyme catalyzes two consequently occurring reactions that require different amounts of oxygen supply, i.e., the ortho-hydroxylation of monophenols to o-diphenols (cresolase activity) and the simultaneous oxidation of o-diphenols to o-quinones (catecholase activity). Spectroscopic studies have also exposed a coupled binuclear copper complex in its active site. (Durán et al. 2002).

10.5.2.2 Laccase

Laccase or para-benzenediol is an oxidoreductase that has copper protein and belongs to a small group of enzymes oxidases. It helps in oxidizing various aromatic compounds such as phenolic dyes, chlorophenols, phenols, benzopyrene, N-substituted by p-phenylenediamines, lignin-related diphenylmethane, organic phosphorus, and non-phenolic beta-O-lignin dimer having the appropriate diminution in the oxygen-to-water ratio. As stated before, many prokaryotes undergo metal metabolism. Similarly, the multi-copper oxidase enzyme that helps in metal metabolism in prokaryotes, with a special prominence on laccase, is briefly discussed in Martins et al. (2015). It is also observed that the prokaryotes have an increased efficiency for oxidation of organic compounds than to metals and are characterized accordingly. These prokaryotes are said to play important roles to confirm their action in intermediary metabolism (Martins et al. 2015). In general, laccases are reported to have four atoms of copper that contribute a chief role in the enzyme catalysis. The distribution of copper atoms is in different binding sites and can be categorized in compliance with its spectroscopic studies (Durán et al. 2002).

10.6 Role of PPO in Plant Immunity

The plants show up their defense mechanisms both actively and passively. The induction and expression of the PPO gene is a part of its defense mechanism (Khodadadi et al. 2016). Many researchers have reported that the expression of PPO is also responsible for plant functions including defense, growth, cell differentiation, and somatic embryogenesis (Grotkass et al. 1995; Kouakou et al. 2009). The PPO expression is certainly high in young plant tissue cells that are most susceptible to insect attacks (Jukanti et al. 2004). Similarly, the PPO was reported to be induced by the JrPPO1 gene present in the walnut that showed a pathogenic defense response against the bacterial blight (Khodadadi et al. 2016). The PPO is ubiquitous among angiosperms and possesses control against pests' activities. The role of PPO in plant defense system is studied by using transgenic tomato plants that overexpressed a cDNA PPO of potato (*Solanum tuberosum L.)* under control of the cauliflower mosaic virus 35S promoter. This resulted in overexpression of PPO and an increased immuno-detectable PPO activity, showing increase in the disease resistance when interacted with the bacterial pathogen like *Pseudomonas* (Li and John 2002). This type of defense mechanism helps the plant from decay-inducing microorganisms or herbivores. The fundamental survival strategies for a seed to germinate after a long period of time include seed dormancy period and its resistance to decay. The research has been done by challenging wild oats (*Avena fatua* L.) caryopses with seeds to the decay-inducing *Fusarium* fungi. The results showed that the dormant seeds exhibited a defense mechanism against the pathogens. Thus this proves to be an essential mechanism for the survival of the seed and longevity or activity in the soil. In the plant tissues, due to wounding, the host defense system along with other enzymes like salicylic acid, jasmonates, etc. triggers the levels of PPO transcript that activates the PPO genes. Generally the PPO is found to be located in the starch

grains and cytoplasm in the undamaged tissue, but during the mechanical bruise, the PPO is said to spread all around to the vacuoles. This is mainly because of the collapse of cell membrane integrity and the bright corroboration of seepage from particular sites (Partington et al. 1999). The cell disruption by the herbivores or other organisms lets the PPO and its substrates to comingle causing the activated defense system: browning reaction.

The mechanisms by which PPO inhibits the herbivores and pathogens include reducing the bioavailability of the proteins and nutrients and producing reactive oxygen species, while the quinone products provide antimicrobial activity and release toxins (Fuerst et al. 2014).

10.7 Genetic Significance

In recent studies, the potato genome is studied and is reported to have nine different PPO-like genes, namely, StuPPO1–StuPPO9. Out of that the first four genes are found to be present in all the potato tissues, while the fifth to eighth genes are not transcribed. But the last gene StuPPO9 is reported to induce PPO gene expression in response to disease defense. Thus research has been done by knocking out this gene by using artificial micro-RNA (amiRNA) technology. This technique proves good for the transformed plants that give less browning phenotype by producing the artificially selected small RNAs of 21 nucleotides in length. Thus RNA is silenced for the PPO expressing gene (Chi et al. 2016). Another set of researchers have experimented same on tuber genome and found the reduced PPO activity along with the less-brown potatoes, which were produced by contemporaneous downregulation of StuPPO2 to StuPPO4 genes, but it showed more abatement when all the genes from *StuPPO1* to *StuPPO4* were suppressed (chi et al. 2014). Few research scholars have studied on the genetically modified tubers (*Solanum tuberosum* L. cv. Estima) to minimize the activity of PPO, and later, the enzymatic discoloration was analyzed for changes in the metabolome either by using liquid (LCMS) or gas (GCMS) chromatography–mass spectrometry. It is observed that the metabolome changes induced over a period of 48 hours (h) by wounding or cutting the tuber into slices were also analyzed using two PPO antisense strands (as PPO) and a wild type (WT) as control (Shepherd et al. 2015). Similarly, the tomato (*Lycopersicum esculentum* L.) genome is reported with seven PPO-like genes at a same locus on chromosome 8 that contains PPO F which is associated with defense mechanism in leaf (Fuerst et al. 2014). These PPO genes congregating in potato and tomato are intended to express their developmental inception via gene duplication (Tran et al. 2012). Apart from the plants, the seed PPOs are also studied, while the hexaploid wheat caryopses are reported to have five genes clustered with introns, at chromosome 2 of all A, B, and D ancestral genomes. In tomato, the family members of PPO in the endosperm and seed coat are said to be activated by the PPO B promoter. Thus it makes cis-elements responsive to phytohormones and cAMP signaling. But still, there is a very limited knowledge on transcriptional regulations of PPO in seed coat and cereals (Feurst et al. 2014). Likewise, four PPO genes are observed in

strawberry genome that showed different expression levels in different tissues. The overexpression of the FaPPO1 genes, which is regulated by abscisic acid or methyl jasmonate, is found to improve the PPO activity in strawberry fruit and is reported to delay the fungus infection process (Jia et al. 2016). The PPO activity and the MAP (modified atmospheric packaging) on the PbPAL1, PbPAL2, and PbPPO1 genes, involved in core browning, are studied with the yali pears under cold storage conditions (Cheng et al. 2015).

Likewise, the variations in the introns of the three different PPO genes in the immature wheat kernels are studied and are reported to sway the transcription of the genes Ta PPO-A1 and Ta PPO-D1. It is also observed that Ta PPO-A1b and Ta PPO-D1b out of the alleles (Ta PPO-A1a, Ta PPO-A1b) of Ta PPO-A1 and (Ta PPO-D1a, TaPPO-D1b) of Ta PPO-D1 are said to express high polymorphism related to polyphenol oxidase activity (Chang et al. 2007). The PPOs in the apricots are very stable that it is expressed even when the mRNA can no longer be detected (Chevalier et al. 1999). Thus this helps us to conclude that the mRNA serves as an excellent indicator for the enzyme function, in the given tissue sample.

Several attempts have been made to clone and characterize the PPO gene present in the red clover, which is responsible during the ensiling of leaves. It is probably the only report to be published on recombination of the PPO that showed restricted activity of expressed protein. (Sullivan et al. 2004). Though there are studies which prove that the PPOs are plastid proteins, the site at which they are present in the tuber of potatoes is still unclear.

A team established clones of transgenic tomato hairy root that overexpress tpx1 gene, by using an atypical procedure of consecutive transformations with *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. This helps in capturing phenolics from the wastewater efficiently. The overexpression of tpx1 gene correlates with increased peroxidase activity, both in vivo and in vitro, in the crude extracts (CE) of transgenic clones obtained from the roots, than in those of the wild-type culture. Thus they are useful in phytoremediation (Oller et al. 2005).

The first successful report of genetic transformation of *Agrobacterium* in the bamboo (*Dendrocalamus hamiltonii*) was done by using a vir gene inducer and PVP was reported by Sood et al. (2014). The study discusses the methods to overcome resistance-like necrosis, due to polyphenol oxidation and formation of waxy layer of the embryos that possess antimicrobial properties, and these methods were found to prevent *Agrobacterium* from attaching to and infecting bamboo, a woody monocot.

Aurone synthase enzyme obtained from *Coreopsis grandiflora* (cgAUS1) catalyzes the conversion of butein to sulfuretin in type 3 copper enzymes, which is a rare example of a polyphenol oxidase involved in anabolism. Site-directed mutagenesis was done at the Cu-A site of AUS1 and the recombinant enzymes were analyzed by mass spectrometry method (Kaintz et al. 2015).

10.8 Chromosomal Location of PPO

Certain studies on hexaploid wheat kernels have proved that out of all the six PPO genes present in them, three of them are expressed out during the growth of kernels (Jukanti et al. 2004). The findings include that the wheat bran lacks transit peptide but expresses matured PPOs placing them into the plastids. But generally, the genetic analysis of the PPO location in wheat kernels is an arduous condition. Jimenez and Dubcosky (1999) reported that a gene from *T. turgidum* coding for PPO was traced in 2D chromosome while the QTLs for PPO in *T. aestivum* indicate that a number of such genes are present on different chromosomes (Demeke and Morris 2002). Genetic mapping or gene tracking also helps in selecting the wheat with lower PPO activities.

The HbPPO1 gene of the rubber tree latex is responsible for secreting the protein that contains the conserved tyrosinase PP01_DWL and PP01_KFDV domains specific to polyphenol oxidases. After analysis of different parts of the tree, *HbPPO1* is reported to be expressed relatively more in the laticifer, than in the leaf and bark (Zhi et al. 2015).

10.9 Wellspring of Polyphenol Oxidases

10.9.1 Mushroom

The PPO in mushrooms is referred to as tyrosinase. As mentioned, this can be converted to diphenols and later to quinones by using catechol/DOPA/tyrosine as substrate. The mushroom tyrosinase is reported to have two heavy chains of 43–45 kDa each and light chains of 13 kDa each. The tyrosinase activity of mushrooms is expressed at different levels in four different growth phases and also differs from tissue to tissue (Hammer 2013).

10.9.2 Walnut

Walnut possesses a single PPO gene (JrPPO1) and generates an exceptionally diverse array of phenolic compounds. This JrPPO1 gene is reported to be overexpressed in all the green tissues and is found to have no response to wounding or methyl jasmonate treatment. This is confirmed by studying transgenic lines of walnut with RNA interference technique by the reducing PPO activity and is observed to develop necrotic spots on the leaves irrespective of the pathogenic infection. The study on the PPO-silenced lines has reported that they play a key role in the Tyr metabolism and, in the absence of PPO, tyramine, a metabolic toxin, is accumulated at higher levels in the walnut leaves. This way, the walnut can be used as a best model to study PPO activity (Araji et al. 2014).

10.9.3 Grapes

PPO from *Vitis vinifera cv*. Riesling is named as *Vv*PPOr. These polyphenol oxidases are naturally occurring type 3 copper proteins in grapes, and they are comprised of the enzymes tyrosinases and catechol oxidases. Catechol oxidase is said to exhibit diphenolase activity (Fronk and Jaeckels 2016).

10.9.4 Ginger

Ginger (rhizobium) is reported to have 0.6–1.5 mg/g f wt or 5–98 unit/mg of phenol content.

10.9.5 Microbes

Polyphenol oxidase from the marine bacterium *Marinomonas mediterranea* (MmPPOA) is a membrane-bound, blue, multi-copper laccase of 695 residues. It possesses peculiar property that distinguishes it from known laccases, such as broad substrate specificity (tyrosinase) to high redox potential (Tonin et al. 2016).

10.10 Production Parameters

10.10.1 Optimization and Purification of PPO from Diverse Genera

PPOs obtained from various plant origins were examined under various parameters like temperature, pH, molecular weight, isoelectric point, and kinetic properties. When catechol was used as the substrate, optimum pH was 7.0. On the other hand, when 4-methylcatechol is used as the substrate, optimum pH was 6.5. The optimum temperature was reported to be 8 °C and was stable below 40 °C. Gisela Palma-Orozco et al. (2011) have reported that the two isozymes of PPO (PPO 1 and PPO 2) were acquired by ammonium sulfate precipitation technique or by hydrophobic ion-exchange chromatography and were found to be monomers. The molecular weight of PPO1 was found to be 16.1 kDa by gel filtration and 18 kDa by SDS–PAGE. The optimum pH and optimum temperature for PPO 1 activity were 7.0 and 350 °C, but the kinetic constant Km was found to be 44 mM and 1.3 mM using catechol and pyrogallol as substrate.

Gao Jia et al. (2011) reported the optimum pH of 7.5 at 0.1 M substrate concentration and temperature of around 20 °C. The Km and Vmax were found to be 3.5 mM and 50 U/min using catechol as substrate. L-Cysteine and ascorbic acid were found to be the most effective inhibitors for sour cherry. Waleed et al. (2009) extracted PPO and homogenized it with potassium phosphate buffer. This mixture

is then followed by acetone precipitation. Km value toward the substrate catechol was found to be 4, 4.16, 1.25, and 2.4 mM for PPOs of apricot, apple, eggplant, and potato, respectively. Wititsuwannakul et al. (2002) have purified PPO by acetone precipitation and later by using the CM Sepharose chromatography, to distinguish between the PPOs of relatively same molecular weights. Pérez-Gilabert et al. (2001) have partially purified the crude extract using phase partitioning technique with Triton X-114 (TX-114) column to obtain double fold purification with 66% activity.

Ridgway and Tucker (1999) reported on partial purification of apple PPO by using a suitable and economical method for industrial usage. Optimized concentration of polyvinyl pyrrolidine (PVP) is used in enzyme extraction process, and the PPO yielded from leaf sample was found to be higher than that from fruit itself. This concludes that the PPOs from leaves are found to be suitable for the commercial application. The partially purified PPO (by 50-fold using the DEAE-12 Sephadex column and Amicon filter unit) had a specific activity of 4.9 μkat mg^{-1} and Km value of 3.6 mM using 4-methylcatechol as substrate. Richardson and McDougall (1997) isolated PPOs from tobacco leaves which were reported to have a molecular weight of 100 kDa when observed with Native PAGE.

10.11 Immobilization of PPO

Generally, enzymes are immobilized either physically or chemically to enhance their stability and to enable the long-term activity of the enzyme. This technique can be done with the help of immobilization matrix. The properties of a good immobilization matrix are that it should be easily available, cost-effective, nontoxic, and biodegradable and must provide large surface area with least diffusion restriction (Arica et al. 2000). High resistance to denaturation, temperature, and organic solvents is attained by immobilizing the enzymes by covalent attachments.

The PPO enzyme can be immobilized on several such matrices as carbon paste, glass beads (Marin Zamora et al. 2007), Nafion membrane, hydrogel, conducting polymers, mesoporous silica materials (Escuin et al. 2017), and organic sources like chitosan, etc. (Dutta et al. 2001). One of them includes the usage of CMC (carboxymethyl cellulose) beads which are obtained naturally from cellulose polymer and can be converted into hydrophilic hydrogels easily via cross linkage with trivalent cations like ferric ions. This is called as “the liquid curing method.” After covalent attachment of epichlorohydrin on the CMC matrix, these hydrogel beads were used for the physical immobilization of polyphenol oxidase (Arica et al. 2000). Chemical immobilization of PPO is done by using a flat membrane of polyamide with a very small pore size of 0.2 m. The dilapidation of phenol in a single-step process can be done by *dead*-end filtration. In this process, the reactive membranes are made by chemically immobilizing the mixed PPO enzymes, directly from the source, on porous micro-filtration membranes. But when the water-soluble species co-deposit along with the enzymes, they reduce their activity. Thus during this condition, a

cross flow filtration is done with osmonics where the solute deposition is hindered on the membrane surface by the hydrodynamic force (Akaya et al. 2002).

Similarly, the so-called blue copper oxidase or laccase was also embedded or immobilized on the silica glass derived from the sol-gel and is proved to improve the enzyme activity manyfold. In this study, the laccase after immobilization showed excellent removal and high stability of the phenols when the 2,4-dichlorophenol (DCP) and 2,4,6-trichlorophenol (TCP) were taken as model compounds (Qiu and Huang 2010). The PPOs were also immobilized on the alginate SiO_2 hybrid (Shao et al. 2009) or the cross linked chitosan-SiO_2 beads to remove aqueous phenol solutions and thus proved efficient in the removal of phenolic compounds from industrial wastewaters (Shao et al. 2007). Several studies have been done on immobilizing the PPOs like the mushroom tyrosinase on several different matrices (like glass beads and cross linkage with cinnamoylated derivatives of D-glucose, D-glucosone, starch, D-fructose, 1,2-*O*-isopropylidene, and d-glucofuranose or partially cinnamoylated derivatives of 3,5,6-tricinnamoyl-d-glucofuranose obtained on hydrolysis of 1,2-*O*-isopropylidene-d-glucofuranose) with addition of many different substrates like L-DOPA, 4-tert-butyl-catechol, dopamine, etc. (MarinZamora et al. 2007).

10.12 Applications of PPO

10.12.1 Industrial Applications

PPOs have a wide range of applications and usage in food processing industries and medicine. They have a vital role in clinical and preclinical diagnosis (Pastore and Morisi 1976). These enzymes are commercially used to remove phenolics from wastewaters, to make them potable. Horticultural products in the food industry suffer a loss of quality and nutritive value in the time between harvesting and consumption. This is due to the oxidation reaction taking place in the endogenous phenolic compounds that results in an inadmissible character called enzyme browning. Deduction of PPO activity has become one of the significant parameters to establish the edibility of fruit and its shelf life. Thus a fast and dependable method has to be carried over to account the strength of the enzyme in various food products. Though polyphenols are generally considered as a drawback in the food industry, they are highly preferred in the food production plants for enhancing the color and aroma in tea and coffee (Motoda 1979).

10.12.2 Applications in Medicine

The antioxidant and antiplatelet activity by the nutraceuticals or the polyphenol-rich nutrients, present in cocoa beans and extra virgin olive oil, are studied for their beneficial properties. It is reported to counteract atherosclerotic complications and reduce the risk of cardiovascular diseases like myocardial infarction or cardiovascular risk factors. It is also reported to indirectly regulate cardiovascular risk factor

such as blood pressure, serum cholesterol, insulin sensitivity, downregulation of oxidative stress, platelet aggregation, and enhancement of endothelial function (Loffredo et al. 2017). Certain evidence implicates that the polyphenols are potential natural therapeutics for Alzheimer's disease (AD) (Zheng et al. 2015). In a study, this experiment is executed by characterizing five anthoxanthin polyphenols, for their ability to reduce amyloid-β (Aβ) oligomer-induced neuronal responses by modulation of oligomerization and antioxidant activity, as well the synergy between these two mechanisms (Pate et al. 2017).

The red wine polyphenols act upon in a multi-target manner, behaving as the nutraceuticals to target neuropathology which is the underlying key mechanism featuring in both Alzheimer's and Parkinson's disease (Caruana et al. 2016).

10.13 Inhibition of PPO

Since the PPO promotes the pigmentation or browning in the food products, due to its reaction with the phenols, it is considered to be a major drawback in the food industry. It serves fruitful in the pathogenic defense mechanism in plants, but it makes food unfit for consumption. Thus the PPO activity must be inhibited in the fruits and vegetables to preserve their properties and nutritional value.

Many researches have been done to restrict the PPO activity in plant products: fruits and vegetables. The expression level of PPO in rubber tree latex was reported to be significantly inhibited by ethylene (Zhi et al. 2015) while L-cysteine was reported to have the highest inhibitory effect on membrane-bound PPO (mPPO) of Fuji apple, followed by ascorbic acid and glutathione (Liu et al. 2015). The broccoli PPO is said to be inhibited by ascorbic acid, citric acid, sodium sulfate, and EDTA (Gawlik-Dziki et al. 2007). Similarly, salicylic acid is said to inhibit the PPO activity in chestnut (Zhou et al. 2015). Yawen et al. (2016) have discussed a few methods like blanching under boiling water or steam, to inhibit the PPO in the lotus roots. Similarly, several methods have been used to inhibit PPO activity of fresh-cut lotus root slices, including PPO passivation with inhibitor; utilization of antioxidant, such as ascorbic acid and sulfur dioxide (Sun et al. 2015); changing reaction and stress condition like pH or water activity (Wang et al. 2009) for the enzyme; isolation of oxygen with different packaging materials (Guo 2013); high-humidity hot air impingement blanching in red pepper (HHAIB) (Wang et al. 2017); or other methods like high-pressure processing (Dong and Kong 2013). These methods can efficiently inactivate the PPO present in the source, providing high shelf life for the food products. Apart from this, the PPO can be thermally inactivated by steam blanching and boiling (Yawen et al. 2016). Though these methods inactivate the PPO activity, the dynamic change that is occurred during blanching is not well studied.

The PPO activity can also be inhibited by dipping it in 2% NaOH for 1 h, which limits the enzyme activity. The prevention of browning that occurs due to any damage is done by treating it with NaOH. This was later found to be due to inactivation of the olive catechol oxidase (Benshalom et al. 1918). The effects of different edible

coatings (like carrageenan and alginate, which are the exopolysaccharide (EPS) from the marine microalgae *Porphyridium cruentum*) along with pectin and carboxymethyl cellulose (CMC) on PPO activity and color change of fresh-cut apple at 4 °C were also studied and are reported to reduce PPO activity at cold conditions (Bertrand et al. 2015).

10.14 Conclusion and Future Aspects

Considering the years of research in the phenol oxidative enzymes, polyphenol oxidase which is reported to have a negative impact on the food industry has a wide application in the industrial sector. It could be due to the fact that the PPOs are involved in general defense response in plants, thus leaving us information that the PPOs are generic but not specialized.

In the future, the bioinformatics approach on these enzymes would certainly aid in identifying these specialized PPOs. Much research has been done on the plant PPO genes and their genomes are sequenced for analysis. Though some of the PPOs are incorporated in defensive mechanism, such as defense of seeds from the pathogens (Fuerst et al. 2014), it is still intriguing that Tran et al. (2012) have located 17 PPO genes out of the total 83 genes that are devoid of chloroplast targeting signal peptide but instead had either a secretory pathway or an unknown intracellular targeting. These provide useful information about the PPOs present as of the case of cell death in walnut PPO discussed under the plant immunity. As a result, the genomics data along with other bioinformatics approach will undeniably aid in better understanding the function of PPO in general and in specialized metabolism.

Thus significant advances on molecular biology, biochemical, and genetic aspects have been made on the PPO enzyme till date, and further study on it will reveal potential uses of PPO in various industries. As said, “The best has to be made out of waste.”

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Xylanases: For Sustainable Bioproduct Production

11

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Abstract

Agricultural waste, the most abundant renewable resource, has gained importance as promising feedstock for the production of biofuels and biobased solvents (butanol, acetone) and enzymes. Enzymatic hydrolysis of hemicelluloses requires synergistic action of multiple enzymes, among them endo-1,4-xylanases (EC. 3.2.1.8) and β-xylosidases (EC 3.2.1.3), collectively known as xylanases, play a major role in the hydrolysis of xylan, the major component of hemicellulose. Though much of research in the past 10–15 years has been on developing technologies for utilization of hemicelluloses, an industrially viable process for commercial production still remains as a major challenge. Xylanases have wide applications in industry. They are used as additive in poultry field, enzyme preparation for hydrolysis of hemicelluloses for bioethanol, biobutanol production, biobleaching of wood pulp in paper industry, food additive in baking industry, and one of ingredient in detergents. This book chapter mainly discusses on recent progress in xylanase production and application with emphasis on heterologous expression, consolidated bioprocessing (CBP) for direct conversion of biopolymers to bioproducts, protein engineering approaches like site-directed mutagenesis, and metabolic engineering of host systems with significance on industrially viable xylanase production.

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Hemicelluloses · Enzymatic hydrolysis · Endoxylanases · β-xylosidases · Heterologous expression · Industrial application

11.1 Introduction

Xylanases are the class of enzymes that catalyze hemicelluloses, present abundantly in lignocellulosic biomass. Lignocellulosic materials are largely generated as waste materials out of agricultural processing. Xylan is a monomer for hemicellulosic polymers like xyloglucan, glucomannan, galactoglucomannan, and arabinogalactan (Gírio et al. 2010). As fossil fuels are depleting at increasing rate, there is an increased concern for developing technologies which are sustainable and are environmentally friendly. Lignocellulosic materials are largely generated as waste materials out of agricultural practices and as by-products from agro-based industries. The utilization of these materials would solve the problem of waste management thereby generating cost-efficient bioproducts. Solvents like biobutanol, enzymes, lactic acid, acetic acid, and bioethanol can be produced from the lignocellulosic biomass which constitutes about 40% of hemicelluloses. Multiple levels of pretreatment methods for lignin removal like acid, alkali pretreatment breaks the lignin and makes available the hemicelluloses and cellulosic polymers for fermentation. To enable cost-effective bioproduct production from lignocellulosic material, multiple enzymes to hydrolyze hemicellulosic and cellulosic polymers are essential. Thus the multiple processing steps and cost of enzymes involved pose a major challenge for large-scale production.

Endo-β-xylanase and β-xylosidase are collectively known as xylanases that hydrolyze the hemicelluloses into monosaccharide units of xylan. Xylanases find numerous applications like biofuel and biobased solvent production, in paper and pulp industry, in textile industry, detergents, and also in baking industry (Beg et al. 2001). Various methods like production of xylanases by microbial hosts and engineering of pathways for xylanase production in non-xylanase producers and engineering of xylanases with improved substrate binding, catalytic activity can pave way for sustainable production of bioproducts from lignocelluloses. Therefore, this chapter will address the recent progress made in xylanase production through heterologous expression, consolidated bioprocessing, metabolic engineering of hosts for maximizing the substrate range, engineering for inhibitor tolerance, and protein engineering strategy of robust xylanase production for industrial applications.

Some of commercially available xylanases with their trade names:

Bleachzyme F	Biocon, Bangalore
Cartazyme HS	Sandoz, UK
Pulpzyme HA, HB, HC	Novozymes, Denmark
Panzea	Novozymes, Denmark
Belfeed BII00	Verenium

Table 11.1 Microbial production of xylanases

Microorganisms	Molecular weight (kDa)	K_m	V_{max}	References
Acidobacterium	41	3.5	403	Inagaki et al. (1998)
Bacillus sp. W–1	21.5	4.5	–	Okazaki et al. (1985)
*Bacillus circulans*WL-12	15	4	–	Esteban et al. (1982)
Bacillus strain BP-23	23	–	–	Blanco et al. (1995)
Bacillus polymyxa CECT 153	61	17.1	112	Morales et al. (1995)
Bacillus sp. NCIM 59	15.8	1.58	4	Devyani (1992)
Acrophialophora nainiana	17.6	–	–	De Aquino et al. (1999)
Aspergillus fischeri Fxn1	31	4.88	55	Raj and Chandra (1996)
Aspergillus sojae	32.7	35	3.75	Kimura et al. (1995)
Geotrichum candidum	60–67	4	50	Knob and Carmona (2010)

11.2 Microbial Production of Xylanase

D-xylose can be used as substrate by the microbial cells for production of chemical fuels, enzymes, and single-cell proteins by enzymatic conversion of xylanases. Due to the complex biochemical structure and heterogeneity of xylan in plant, its breakdown requires the action of several hydrolytic enzymes with distinct modes of action and more diverse specificity. Thus xylanases, isolated from various microbial sources, show different kinetics toward the substrate. The xylanases isolated from a range of sources are summarized in Table 11.1.

11.3 Regulation of Xylanase Biosynthesis

Large production of xylanases was first reported in *Cellulomonas flavigena*, using xylan as substrate. As high molecular weight xylans are difficult to be uptaken by the microbial cell, there was need for induction of xylanase production by simpler xylan fragments (Bastawde 1960; Kulkarni and Rao 1996). Production of xylanase can be induced by other compounds like various xylooligosaccharides, L-sorbose, lignocellulosic residues, and xylose. It was reported that L-sorbose induces the production of xylanase in *Sclerotium rolfsii* (Sachslehner et al. 1998) and *Trichoderma reesei* PC-3–7. Manikandan et al. (2006) reported a twofold increase in xylanase production with *Staphylococcus* sp. SG-13 in a medium containing an upper liquid layer and a solid lower layer of agar with wheat bran substrate (Manikandan et al. 2006). The regulatory mechanism of xylanases has not been studied till now.

11.4 Heterologous Expression of Xylanases

11.4.1 Bacterial Systems

Enzyme for commercial application requires high expression levels along with a cost-efficient and economically viable process. Thus, in order to achieve this, engineering of microbial hosts for heterologous and secretory expression is essential.

For large-scale industrial production of xylanases, bacterial hosts like *E. coli*, *B. subtilis*, and *Lactobacillus* are widely exploited. *E. coli* offers several advantages like ease for genetic manipulation, growth on inexpensive media, ease for scale-up and downstream processing, and less processing time. Despite these facts, expression of recombinant proteins to high titers is hindered by factors like lactate accumulation, lack of posttranslational modification like glycosylation, disulfide bond formation, and mostly protein expressed as inclusion bodies.

Recombinant expression in *Lactobacillus* cells (GRAS) makes it easier to be used in dairy and food industries. *Lactobacillus* xylanases were widely used for releasing fermentable sugars from silage preparations. Recombinant expression from an alkalophic and thermophilic *Bacillus* NCIM 59 for the production of xylanase showed sixfold increase compared to that of its wild host (Kulkarni et al. 1999). Single-point mutations to XynA gene from *B. subtilis* (N181R, N32D, S27E, N54E, and S22E,) were generated by multiplex PCR-based recombination (MUPREC) to create mutants for generation of combinatorial library. All the mutants were expressed in *E.coli BL21*(*DE3*) host among which the double-mutant S22e/N32D showed increase in specific activity at 55 °C compared to that of its native type (Wang et al. 2014b).

Large number of novel thermophilic bacteria and Achaea genomes are sequenced for the hunt of secondary metabolites with high tolerance to extreme temperature and alkaline conditions. A gene coding for xylanase from the *Thermotoga thermarum* was cloned and expressed in *E.coli BL21* (*DE3*). A*mino* acid hydrophobic cluster, three-dimensional structure analysis, and sequence homology revealed that the xylanase comes under the glycoside hydrolase (GH) family. The enzyme exhibited high thermostability at temperature 55–90 °C with the addition of 5 mM Ca^{2+}, and high specific activity of 148.5 U/mg was achieved (Shi et al. 2013).

The recombinant xylanase screened from compost soil using metagenomic and combinatorial library analysis exhibited huge thermostability with half-life period of 2 h at 80 °C and 15 min at 90 °C making it feasible for exploitation in paper and pulp industry (Verma et al. 2013).

11.4.2 Yeast Expression System

Yeasts are the most preferred hosts for expression of heterologous gene to high titers in grams per liter. They are highly preferred over bacterial expression systems for their tremendous ability to execute eukaryotic posttranslational modifications and their ability to achieve high cell densities along with secretory expression. Moreover,

yeasts are recognized as GRAS organisms and are exploited for production in food and pharmaceutical industry. *Pichia pastoris*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, *and Hansenula polymorpha* are the widely used hosts for heterologous protein expressions (Verma and Satyanarayana 2012).

Yeasts also possess limitations for high-level expression due to factors like hyper-glycosylation and endoplasmic reticulum-associated stress response. ERAD (endoplasmic reticulum-associated degradation pathway) and UPR (untranslated protein response) are elicited when heterologous proteins are overexpressed. To overcome these challenges, proteins are co-expressed with molecular chaperones like Pdi and Kar2p to relieve ER stress (Gasser et al. 2008).

Recombinant *Saccharomyces cerevisiae* co-expressing *Trichoderma reesei* xylanase II (*XynII*) and *Aspergillus niger* β-xylosidase (*XlnD*) genes were expressed with the help of promoter ADH 2 (alcohol dehydrogenase). The recombinant *S. cerevisiae* strains produced β-xylanase with the activity of 1577 nkat/mL (Wang and Xia 2008).

Surface-engineered minihemicellulosome on recombinant *Saccharomyces cerevisiae* strains capable of combining xylan hydrolysis, hydrolysate fermentation, and hemicellulase production into a single step was reported. These strains displayed uni-, bi-, and trifunctional minihemicellulosomes which contain a miniscaffoldin and three chimeric enzymes. The miniscaffoldin from *Clostridium thermocellum* consisted of three cohesion modules and was adhered to the cell surface through a-agglutinin adhesion receptor in *S. cerevisia*e. Up to three types of hemicellulases, endoxylanase (XynII), xylosidase (XlnD), and an arabinofuranosidase (AbfB), were arranged onto the miniscaffoldin. The resulting quaternary trifunctional complexes showed a hydrolysis of arabinoxylan with 0.3 g/L ethanol yield when compared to bifunctional minihemicellulosomes (Sun et al. 2012).

Methylotrophic yeast *Pichia pastoris* owing to its advantages like strong inducible promoters of alcohol oxidases (AOX), growth on methanol as carbon sources, and ability to grow at high cell densities is widely used for heterologous gene expression for industrial application. A *XynA* gene from *Thermomyces lanuginosus* was genetically modified and expressed in *Pichia pastoris* with (glyceraldehyde 3-phosphate) GAP promoter. The production was scaled up to a 5 L fermenter, and highest productivity of 138 IU/mL was obtained (Birijlall et al. 2011).

The major challenge for heterologous enzyme production using hemicellulosic and cellulosic sugars goes into the cost of multiple enzymes required for the synergistic action. A recombinant *Pichia pastoris* host co-expressing phytase and xylanase in a single vector using cis-acting hydrolase elements was developed. The genes were linked by the 2A-peptide-encoding sequence and were fused in frame with the α-factor secretion signal. Both phytase and xylanases were expressed as individual functional proteins with higher specific activity (Roongsawang et al. 2010).

Microbial oils are considered as alternative feedstock to plant oils for production of biofuels. *Yarrowia lipolytica*, oleaginous yeast, is known to accumulate lipids intracellularly by metabolizing sugars. They are extensively studied for engineering of hemicellulolytic enzymes for direct conversion of biofuels. The XynII and XlnD

genes were expressed in *Y. lipolytica* and were observed that endoxylanase and exo-xylosidase activity of 14 nkat/mL and 1396 nkat/mL, respectively, was achieved comparable to that of enzyme production in *S. cerevisiae* (Wang et al. 2014a).

Heterologous expression of xylanase genes from *Dictyoglomus thermophilum*, *Neocallimastix frontalis*, *Thermotoga*, *and H. insolens* species in *K. lactis* showed highest productivity of 130 mg/mL (Van Ooyen et al. 2006). With an attempt for the hunt of novel xylanases, a quantitative analysis of gene expression profiles for the xylanases duringfeeding cycle in the sheep rumen was studied. Six xylanase gene expression profiles were detected at all time, and a complex trend of dynamic gene expression was observed, depending on ruminal conditions. Thus novel xylanases genes with high expression profiles can be exploited for large-scale production (Li et al. 2013).

11.5 Engineering for Inhibitor Tolerance

Acetic acid, formic acids, furfurals, and phenolic compounds which are released during the pretreatment process inhibit the fermentation process (Lee 1997). Depending on the type of biomass substrates and pretreatment methods, the concentration of acetic acid found in the lignocellulosic hydrolysate varies (Klinke et al. 2003; Tomás-Pejó et al. 2010). Though the concentration of formic acid is generally lower than that of acetic acid, it causes severe toxicity to *S. cerevisiae* (Hasunuma et al. 2011; Martin et al. 2007). Pentose sugar and hexose sugar hydrolysis releases 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural; 5-HMF), respectively. Vanillin, ferulic acid, and 4-hydroxybenzaldehyde are phenolic compounds formed during lignin removal.

The intracellular anion accumulation and uncoupling are the common limitations caused by weak acids. Intracellular anion accumulation inhibits the metabolic activity of the cell causing the efflux of electrochemical gradient across the plasma membrane (Mira et al. 2010). Acetic acid inhibits intracellular RNA, DNA synthesis, and the glycolytic enzymes (Pampulha and Loureiro-Dias 1990). Furfurals and 5-HMF inhibit alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), and pyruvate dehydrogenase (PDH) (Modig et al. 2002). GAPDH in yeast cells are inhibited by furfurals (Banerjee et al. 1981).

The strategies employed for engineering inhibitor tolerance are:

- Directed evolution for improving the tolerance of host
- Mutagenesis and genome shuffling approach
- Overexpression of enzymes in metabolic pathway
- Metabolic engineering for inhibitor tolerance

A *Saccharomyces cerevisiae* strain capable of fermenting D-xylose was developed through evolutionary engineering approach. The separate hydrolysis and fermentation with wheat straw hydrolysate yielded 5.8% (v/v) ethanol titer. Directed

evolution of ethanol red strain with increasing xylose concentration was capable of utilizing xylose at a rate of 1.1 g/g dcw (Demeke et al. 2013).

The xylose-fermenting *S. cerevisiae* strain, engineered for inhibitor tolerance by directed evolution, showed enhanced ethanol production than the native strain with sugarcane bagasse as substrate (Martin et al. 2007). High ethanol titers from xylose, in the presence of acetic and formic acid, were obtained in *S. cerevisiae* host by adopting multiple strategies like overexpression of homologous or heterologous ADH1, ADH 6, and ADH7 genes (aldehyde dehydrogenases), mutation of FPS1 gene encoding for aquaglyceroprotein uptake and efflux, overexpression of formate dehydrogenase (FDH) for detoxification of formic acid, and pentose pathway gene TAL1 (Hasunuma et al. 2011). Laadan et al. (2008) reported an ADH1 variant resistant to furfurals.

11.6 Consolidated Bioprocessing, Cell-Surface Display

The multiple processing steps of lignocellulosic biomass like pretreatment, saccharification, and fermentation add up to the cost of fuels as well as enzymes produced from lignocellulosic biomasses compared to that of cornstarch or molasses substrates. The hemicellulolytic and cellulolytic enzyme pathways are engineered and displayed on the cell surface of fermentative host for consolidated bioprocessing (CBP), which combines three different processes in a single step, thus reducing the cost as well as enabling the host for utilization of broad range of substrates. CBP biocatalyst is made to express hemicellulosome and cellulosomes from either fungi or bacteria in the yeast *Saccharomyces cerevisiae* cell surface through glycosyl phosphatidylinositol anchoring system (Kondo and Ueda 2004). This enables the reutilization of the host cells without loss of activity (Ueda and Tanaka 2000). A yeast cell surface co-display of *A. aculeatus* β-glucosidase 1 (BGL1) and *T. reesei* endoglucanase II (EGII), capable of utilizing β-glucan from barley, was developed. The yeast was able to ferment 45 g/l β-glucan, with ethanol productivity of 0.48 g/g of glucan which corresponds to 93.3% yield. SSF of recombinant yeast cell utilizing phosphoric acid-swollen cellulose as substrate co-displaying three cellulolytic enzymes, *T. reesei* EGII and *cellobiohydrolase* II (CBHII) and A. *aculeatus* BGL1, on the cell surface yielded 0.45 g/g of ethanol with 88.5% of the theoretical yield (Fujita et al. 2002).

The CipA1 and CipA3 domain have cohesin Coh1, Coh2, and Coh3 tethered to cell surface through AgaI and AgaII. Endoxylanases, xylosidases, and arabinofuranoside genes were displayed as miniscaffoldin on cell surface and expressed in same one-to-one ratio via non-specific cohesin-dockerin interactions.

A cellulose-degrading thermophilic anaerobic bacterium, from a Himalayan hot spring (*Clostridium* sp. DBT-IOC-C19), exhibited broad substrate specificity over various cellulosic and hemicellulosic substrates to produce ethanol, acetate, and lactate (Singh et al. 2017).

11.7 Protein Engineering

Paper and pulp industries require high temperature and alkali-tolerant xylanases. Kraft cooking, a unit operation in paper industry, operates at 170 °C with NaOH and sulfides. The major challenge exists as the xylanases from thermophiles were stable at high temperature with activity at 95 °C, but they are not alkali tolerant and operate around neutral to acidic pH (6.0–7.5). Protein engineering for alkali and thermostable xylanases is of significance in paper and feed industry. Analysis of catalytic domains, thermostabilizing domain, substrate-binding domains, critical amino acids, and covalent and non-covalent interactions is prerequisite for designing the xylanase of choice. The following strategies are adopted to engineer xylanases (Verma and Satyanarayana 2012):

- Site-directed mutagenesis
- N-terminal substitutions with thermophilic xylanase residues
- C-terminal addition to enhance thermostability
- Error-prone PCR
- Gene site saturation mutagenesis
- Disulfide bonds to increase the stability

11.8 Site-Directed Mutagenesis

The crucial amino acid at particular position defines the behavior and function of the enzymes. Site-directed mutagenesis is widely used to alter the stability of protein and improve substrate specificity and catalytic activity. The role of an amino acid can be studied by using inhibitors to particular amino acids like N-bromosuccinimide (tryptophan), phenylmethylsulfonyl fluoride (serine), iodoacetate, DTT, and mercaptoethanol (cysteine). Phe 4, Trp 6, and Tyr 343 in G10 family xylanases were important for proper folding of the protein. Deletion of Phe 4 resulted in complete loss of activity. Increased thermal stability in xylanases from *Bacillus cereus* was obtained by tyrosine to asparagine substitution at position 52. Xylanase from *A. niger* showed 18–20-fold increase in thermostability by replacement of arginine to serine and threonine (Verma and Satyanarayana 2012). The significant point mutation in various xylanase genes from different sources is summarized in the Table 11.2.

11.9 Application of Xylanases

Commercial xylanases are used in biobleaching, paper and pulp industry, baking industry, and biorefineries and for lignocellulosic ethanol, biobutanol, and xylitol production. They are widely used in production of sugar syrups and xylooligosaccharides. Xylanases with high alkali and thermostability are of great need for the paper and pulp industry. The harsh alkali pretreatment conditions pose a serious

Table 11.2 Protein engineering approaches (Verma and Satyanarayana 2012)

Organism	Engineering approach	Inference	References
B. circulans	Asn 35 to Asp	Increase in catalytic activity	Li and Wang (2011)
T. reesei	Enhancement of C-terminal processing	Half-life increased by 63 min	Turunen et al. (2001)
A. niger	Deletion of C-terminal	Increase in thermal stability by 6 degrees	Liu et al. (2011)
T. lanuginosus	Error-prone PCR A54T	Alkali stable	Gaffneya et al. (2009)
C. japonicus	Error-prone PCR	Increase in thermal stability	Andrew et al. (2005)

issue on the environment and personnel; hence the industry looks for alternative biotechnological methods which would reduce a part of chemical treatment processes. Thus xylanase treatment increases the efficiency of lignin removal from hardwoods and softwoods and provides the best alternative to traditional pulping methods which uses chlorine dioxide and sodium hydroxide (Walia et al. 2013).

For the sustainable biofuel production, xylanases play a major role in saccharification of hemicelluloses, thus increasing the saccharification efficiency. Xylanases and phytases are used as animal feed to improve the digestion of lignin and hemicelluloses in animal feed. Xylooligosaccharides (XOS) find potential application in food and beverages. XOS sugars are used in prebiotic formulation, in beverages like soymilk, tea, and coffee, in pastries, and in special formulations for elderly people and children. Xylanases are widely used in baking due to their property to interact with gluten. Xylanases improve the bread quality and are also used in clarification of fruit juices. Xylanases are commercially used for xylitol production. Xylitol is a five-carbon polysaccharide having sweetness comparable to sucrose; hence it's widely used in the food and pharmaceutical industry due to its properties like low viscosity and cooling effect produced when dissolved in solution. Thus the growing demand of xylanases emphasizes the need for large-scale production with utilization of biobased materials (Laadan et al. 2008). The common industrial applications of xylanases are summarized in the Table 11.3.

11.10 Metabolic Engineering

To engineer microbes for industrial production of bioproducts from lignocelluloses, metabolic performance of the cells needs to be increased by systems metabolic engineering. Random mutagenesis and screening approach can lead to unknown genotypic and phenotypic changes in genome when the process conditions are altered. Systems metabolic engineering has emerged as prominent tool for engineering at genome level, increasing substrate range, and engineering inhibitor

Table 11.3 Industrial applications of xylanases

Source	Industry	Application	References
Aspergillus niger IBT 70	Baking	Increase in dough volume when 1200 U/kg of dough is added	Butt et al. (2008)
Aspergillus foetidus	Baking	13% increase in dough volume was obtained with xylanase addition	Butt et al. (2008)
Thymus lanuginosus	Baking	Had 40% increase in dough volume as the enzyme from the source had highest specific activity	Butt et al. (2008)
	Food	Along with amylases and glucose oxidases for production of high-fructose corn syrup and in production of xylose oligosaccharides	Sachslehner et al. (1998)
Clostridium cellulans CKMX1	Biobleaching	Alkali-tolerant and thermostable xylanases showed kappa number reduction of 0.5 and 0.8 according to ISO standards	Walia et al. (2017)
Aspergillus niger	Biobleaching	20% reduction in chlorine treatment with increase in the brightness of sheets	Buchert et al. (1994)
Aspergillus flavus	Animal feed		
Cellulomonas flavigena	Food	Clarification of fruit juices and in processing of fruit pulps	Kulkarni et al. (1999)
Candida rugosa	Food	Microbial xylitol production from xylose with 65% xylitol yield	Xi et al. (2010)
Petromyces albertensis	Food	Highest xylitol production with yield of 0.4 g/g of xylose	Xi et al. (2010)

tolerance, thereby increasing the scope of microbes for producing wide range of products from biobased chemicals to isoprenoids. Systems metabolic approach includes several techniques like in silico modeling and simulation, high-throughput screening, genomics, transcriptomics, proteomics, metabolomics, fluxomes, synthetic regulatory circuits, gene synthesis, and enzymes and pathway engineering (*Ledesma*-Amaro and Nicaud 2016).

Common metabolic engineering approaches are:

1. Engineering the microbes for cheaper substrates and reducing pretreatment at upstream processing
2. Metabolic engineering to improve fermentation

   Engineering the desired pathways, downregulation of competitive pathways, and channeling flux towards product formation can increase the yield and productivity.
3. Engineering of microbe to enhance downstream process by reducing the byproduct formation by the following approaches

(i) Surface display or extracellular secretion of enzymes to convert biopolymer to monomers
(ii) Transport mechanisms
(iii) Degradation pathway
(iv) Central and specific metabolic pathways

Depending on the type of biomass used, the organism activates a particular metabolic pathway and contributes to energetics and redox cofactors at varying levels. Inward and outward fluxes are adjusted by overexpressing or knocking out key bottlenecks and regulatory proteins.

(v) Tolerance to toxic compounds

The major challenge in biofuel production from lignocellulosic biomass is that ethanol producers like *S. cerevisiae* and *Z. mobilis* are not five-carbon utilizers and the major five-carbon-utilizing bacterial strains are not ethanol producers. Hence metabolic engineering attempts were made to engineer ethanol-producing pathway in *E.coli* hosts and an alternative approach of five-carbon utilization pathway for direct single-step ethanol synthesis in *S. cerevisiae* and other classical yeasts.

To control the redox imbalance, NAD-dependent FDH was overexpressed in *E. coli* which resulted in increase in cell density and a 22-fold increase in ethanol production compared to that of native *E. coli* strain (Berríos-Rivera et al. 2002).

11.11 Conclusion

Xylanases, the most studied hemicellulolytic enzyme, can be exploited for a wide range of industrial applications. In order to meet the performance of the enzyme for industrial needs, various enzyme engineering approaches have to be adopted to arrive at a robust enzyme. Protein engineering approach can be used to design xylanases with desired properties. Metabolic engineering with systems biology approach has opened up multiple strategies like cell-surface display of hemicellulosomes and cellulosomes for increasing wide range of substrate utilization and consolidated bioprocessing for direct conversion of biopolymers to products with minimal by-products. Combination of these methods with future developments would promise to enable the vision of bioprocessing for sustainable development.

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12 Inulinase: An Important Microbial Enzyme in Food Industry

Anand Mohan, Bableen Flora, and Madhuri Girdhar

Abstract

Inulinases are industrial food enzymes which have earned vast attention recently. Inulin and inulin-containing materials are sustainable, economical polymeric carbohydrates which can be easily hydrolysed by microbial inulinases into fructose, glucose and inulooligosaccharides. The inulinase gene can also be cloned and can be used in the production of bioethanol, single-cell oil, and single-cell protein utilizing inulin as the substrate in many species of yeast. The utilization of inulin is immense for the production of monomeric fructose units, and it has replaced starch in many food industries with multiple applications. Plants like agave, asparagus, coffee, chicory, dahlia, dandelion, garlic, Jerusalem artichoke, etc. are richest source of inulin. Inulin showed encouraging biorefinery approach in which inulin-containing waste, produced with the help of microorganisms has been used to yield biofuels including renewable gas, renewable diesel and further for the production of electricity.

Keywords

Inulinase · Bioethanol · Single-cell oil · Inulooligosaccharides

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12.1 Introduction

Inulin is natural polysaccharide products of various plants like dahlia, leeks, garlic, onion, asparagus, Jerusalem artichoke, etc. It is also found as a reserve food source in the tubers as well as roots of plants such as Jerusalem artichoke, chicory and Dahlia.

Inulins are used in the production of biofuels as bioethanol. It has many other applications also as single-cell oil production which can be a substitute for the fats and oils. Inulinase enzymes catalysed the production of high-fructose syrups, used as sweetening agents. Inulinases plays important role in the production of inulooligosaccharides-low caloric saccharides acting as growth factors for many intestinal floras. Fructose is found to be safe as compared to sucrose due to its beneficial effects in diabetic patients, elevates the iron absorption in children and has higher sweetening capacity. Fructose syrup was firstly obtained from corn starch, but nowadays both fructose and fructooligosaccharides are vital products of inulin. Not only in food industry but it has major impact in various pharmaceutical industries. Bowel diseases like IBS, diabetes, obesity and many digestive problems can be cured by inulin intake. It also has shown other health benefits due to low calorific value, cholesterol reduction and selective utilization by intestinal bacteria. It is widely distributed in plants and can be obtained from daily consumed vegetables, fruits and cereals like onion, wheat, chicory, banana and dandelion roots.

At the global level the inulin market showed a great measure of growth among health issues and has been considered one of the best ingredients in the dairy products keeping unhealthy at a bay. The major sellers of inulin are Coscra, Novabio rubber, Jarrow Formulas, Now Foods, Green Labs LLC, Sensus, etc. There are various ongoing projects worldwide, which are highly attracted by multitude characteristics of inulin and getting most of it from different sources. The Nova Green project is exposed to the cultivation of Jart and the processing of Jart into xylitol, inulin and biochar along with AITF in Eastern Alberta.

12.2 Structure of Inulin

Inulin is made up of fructose units that are linked together by $\beta(2 \rightarrow 1)$ bond and at terminal end contains D-glucose linked to the fructose by $\alpha\ (1 \rightarrow 2)$ bond, belongs to hydrolases group of enzymes. The general formula for inulin is $C_{6n}H_{10n+2}O_{5n+1.}$

Inulinase is hydrolysing to fructose, glucose and inulooligosaccharides (IOS) (Chi et al. 2010). Inulinase is further divided into two types depending on their mode of action (Fig. 12.1).

12.2.1 Exoinulinase

It catalyses the removal of terminal fructose molecule. They can also show invertase activity along with hydrolytic action.

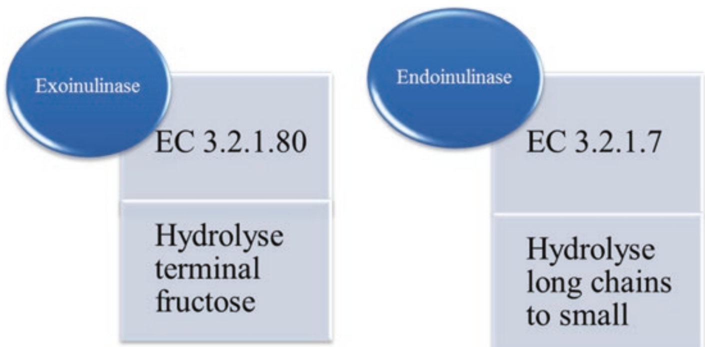

Fig. 12.1 Two types of inulinase with their Enzyme Commission (EC) number, based on the chemical reactions they catalyse

12.2.2 Endoinulinase

It hydrolyses the long chains into smaller molecules to form inulotriose, inulotetraose and inulopentose. It lacks invertase activity.

Microorganisms are found to be the best sources for commercial production of inulinases, due to their uncomplicated cultivation and their essential applications in various industries. Microorganisms from *Aspergillus*, *Penicillium*, *Kluyveromyces*, *Cryptococcus*, *Pichia*, *Bacillus*, etc. have been proved to be high producer of inulinase.

Exoinulinase and endoinulinase have different active sites and contrast activities (Fig. 12.2). Various experiments for structural differences provide a solid groundwork which explicit molecular basis of enzyme activity. This can be helpful in future studies of mutagenic experiments.

12.3 Forms of Inulin

Inulin can be obtained from different sources with variety of applications. They are available in different forms (Fig. 12.3) and are as follows.

12.3.1 Native

The native form of inulin can be obtained from natural sources, as it is present as storage material in tubers of onion, garlic, dahlia, etc. Native inulin is a rich source for dietary fibres and helps to reduce weight and indigestion problems. Different parts of plants have been used for extraction of native form of inulin as Root tubers, bulbs, grains, rhizome, etc.

Endoinulinase (acts randomly)

Exoinulinase (acts sequentially)

Fig. 12.2 Different active sites of endo- and exoinulinase with contrast activities

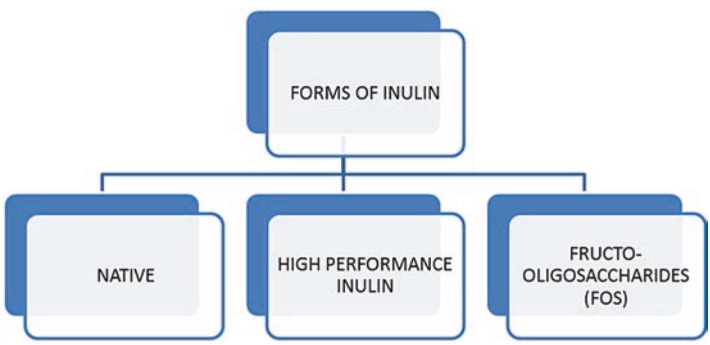

Fig. 12.3 Three different forms of inulin

12.3.2 High-Performance Inulin

Such inulin can be obtained by removal of shorter molecules from inulin. It helps in relieving constipation, acts as antioxidant and also prevents diabetes type 2.

12.3.3 Fructooligosaccharide

It is the form of inulin which contains small inulin molecules synthesized from table salt. These are oligosaccharides of fructose produced by fructosyl transferase (FTase). FOS act as a substrate for intestinal flora and also improve the gastrointestinal tract health. FOS and inulin also promotes the uptake of calcium in both animal and human gut. FOS showed exotic nutritional and practical traits which improve the shelf life and taste profile of various food items. The use of inulin or inulin-derived fructans has been used in dairy and bakery products and took the place of sucrose enormously.

12.4 Production of Inulinase with Various Methods and Sources

Inulin is a soluble fibre and carbohydrate reservoir in the roots and tubers of plants. These inulin sources have been got into consideration for the enzymatic production of fructose syrups and as a hidden feedstock for bioethanol production. Inulin is of great interest because it is a comparably economical and rich substrate for the production of rich fructose syrups (Vandamme and Derycke 1983). Fructose and fructooligosaccharides have become an important area of research, pertaining to their beneficial properties such as controlling diabetes, escalating iron absorption in children and having a higher sweetening capacity as compared to sucrose (Pawan 1973). In addition, fructose is richly soluble as compared to sucrose, in regard to viscosity, and at low levels, it can be metabolized without the need for insulin (Fleming et al. 2009). Microbial inulinase undergoes enzymatic hydrolysis of inulin which leads to the production of fructose (sweetener) and inulooligosaccharides. Inulin is one of the most ample nonstructural polysaccharides extensively dispersed in plants and awaits careful application. Increased Inulinase production due to their elevated use as the substitute for an alternative and healthy sweetener prompt their industrial interest. Inulinases have been produced through various microbial sources including yeasts, moulds and fewer bacterial species also. Recently, in the heat of the moment, researchers are trying to find a novel inulinase producer using crude plant inulin and agro-industrial media for industrial-level production of inulinase in submerged as well as solid-state fermentation (Vandamme et al. 1983). Nowadays the microbial inulinase converged the interest of researchers. Inulinases can be produced by many microorganisms as host cell including fungi, yeast and bacteria. Inulinase acts as catalyst in the hydrolysis of inulin and forms D-fructose (fructose syrup), which is a vital nutrient of human diet (Pandey et al. 1999).

Currently new competent producers of inulinases from microorganisms were isolated from Jerusalem artichoke tubers grown in Thailand which has been characterized as *Aspergillus niger* TISTR 3570 and *Candida guilliermondii* TISTR 5844. These microbial inulinases found to hydrolyse inulin to fructose as the prime product. With the starting inulin concentration of 100 g l^{-1} and 0.2 U g^{-1} of substrate, 37.5 g l^{-1} of fructose has been produced in 20 h at 40 °C when *A. Niger* TISTR 3570 inulinase was used as the catalyst. Under identical conditions, the yeast inulinase produces 35.3 g l^{-1} of fructose in 25 h. The fructose yield was 0.35 g^{-1} of substrate (Sirisansaneeyakul et al. 2007). Microorganisms found to be outstanding source for industrial production of inulinase due to its ease of cultivation. Till now, it has been reported that *Aspergillus* sp., *Penicillium* sp., *Bacillus* sp., *Clostridium* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Staphylococcus* sp., *Xanthomonas* sp., *Kluyveromyces* sp., *Cryptococcus* sp., *Pichia* sp., *Sporotrichum* sp. and *Candida species* are the prominent sources of inulinase (Gao et al. 2007). It has been studied that inulinase can be abundantly produced from yeast strains than other strains like bacteria and fungi. *Kluyveromyces fragilis*, *Cryptococcus aureus*, and *Kluyveromyces marxianus* have been investigated for producing industrially agreeable output of the enzyme (Gong et al. 2007; Sheng et al. 2009). Response surface methodology (RSM) is the procedure used to elevate the cultivation circumstances as well as medium concentration for the production of inulinase by the mutant strain M-30 in the submerged fermentation (Yu et al. 2011). Superlative yield of inulinase (55.4 U/ml) was investigated by isolated strain of *K. marxianus* YS-1 in a stirred-tank reactor at an agitation rate of 200 rpm with the aeration of 0.75 volume/volume per minute and fermentation time of 60 h (Singh et al. 2007). Whereas inulinase activity was found to be 50.2 U/ml with the same yeast strain under agitation (200 rpm) and aeration (0.75 volume/volume per minute) at 30 °C after 60 h of fermentation in 1.5 litre of fermentor while using organic inulin (4.0%) obtained from root tubers of *Asparagus officinalis*.

In one of the works on yeasts, *Kluyveromyces marxianus* CBS 6556 was chosen for studying the parameters that influence economical production of inulinase. The selected yeast has superior properties for the growth at elevated temperatures ranges from 40 to 45 °C, substrate specificity for inulinase production. A generous part of the inulinase produced by *K. Marxianus* was attached with the cell wall, and through chemical treatment, the enzyme can be separated out. Inulinase showed progressive reaction with sucrose, raffinose, stachyose and inulin as substrates and exhibited an S/I ratio (relative activities with sucrose and inulin) of 15 at defined conditions. The enzyme activity found to be inversely related to chain length. In other words researchers found that with the increase in the length of chain of the substrate, enzyme activity decreases (Rouwenhorst et al. 1988). Inulin has been also produced from thermophilic bacteria. Four strains of thermophilic bacteria were isolated through enrichment in batch and continuous culture with inulin as the basic source of carbon and energy. Although the growth temperature for inulin was found between 40 °C and 65 °C, the optimum growth temperature is 58 °C. All strains were capable of fermenting a large number of sugars and organic fermentation products including formate, acetate, ethanol, lactate, H_2 and succinate. Synthesis of inulinase in batch

culture showed complementary growth in continuous culture and concluded that the enzyme was completely cell bound (Drent et al. 1991). Sugarcane bagasse an agro-industrial residue used as carbon source from which exoinulinase is (beta-D-fructan fructohydrolase) produced using *Aspergillus terreus* CCT4083. The purified enzyme obtained was 57 kDa on SDS-PAGE and 56 kDa on gel-filtration chromatography. Then, D-fructose was produced as the main product by hydrolysis by purified enzyme, along with highest activity at pH 4.0 and temperature of 60 °C. There was a comparative study of inulinase obtained from *A. Terreus* with inulinase isolated from other microbes. The exoinulinase showed thermostability and its efficient capability to produce pure D-fructose, which leads to various applications like production of high-fructose syrup (Coitinho et al. 2010). A recombinant inulinase-producing *Saccharomyces cerevisiae* strain was build up which lacks gene for fructose uptake by interrupting hxk1 and hxk2 genes. Then incorporated inulinase gene into *S. cerevisiae* was cloned from *K. cicerisporus*. Extracellular inulinase activity of the recombinant mutated *S. cerevisiae* strain reported 31 U/ml after 96 h growth. If Jerusalem artichoke tubers were used as basic component in the growth medium, then recombinant yeast formed nearly 9.2% (w/v) in the fermentation broth with only 0.1% (w/v) glucose left after 24 h incubation (Yu et al. 2011). *Aspergillus niger* isolated from soil sample also found to produce inlunase. For maximum production inulinase on corn steep liquor, maltose medium without inulin at 28 °C was also directed. Extrainulinase as well as intracellular inulinase were displayed identical pH and temperature optima with maximal activity at pH scale of 4.3–4.4 and temperature range of 55–56 °C. The high temperature yields more fructose as well as prohibits the microbial contamination in bioreactor. Besides controlling colour formation, the low optimum pH further prevents undesirable chemical side reactions. Crude inulin (chicory) extracts are hydrolysed rapidly than pure inulin. Besides inulin (100% hydrolysis), sucrose (45%) and raffinose (20%) that can also be hydrolysed without releasing oligomers or sucrose were obtained. This reveals that the *A. Niger* enzyme is an exo-acting inulinase. Due to all these properties, *A. Niger* inulinase has become an commercially interesting enzyme for preparing pure fructose from inulin-containing agricultural crops (Vandamme and Derycke 1983). Various optimization methods have been used for the production of inulinase. The pH and temperature values of the cultivation medium are important optimized parameters to enhance the production of inulinase (Dinarvand et al. 2017). FOS has been extensively used in dairy, confectionary and other food products; so its production is being reformed for low-cost production by immobilization of inulinase in polyurethane foam (de Oliveira Kuhn 2016).

12.4.1 Effect of Different Optimization Parameters on Inulinase Production

Inulinase production shows various optimization factors which effect its production. The various factors including carbon source, nitrogen source, pH, temperature, aeration and agitation has great impact on the production of inulinase. Many carbon

Table 12.1 The description of industrial usage of inulinase with their sources from which inulinase and inulin has been obtained and their high-end product into which it has been further processed

Source of inulin	End product	Strain source of inulinase	References
Agave juice	Ethanol	*Kluyveromyces marxianus*	López-Alvarez et al. (2012)
Chicory roots	Fructooligosaccharide	*A. niger*	Kango and Jain (2011)
Yarrowia lipolytica	Erythritol and citric acid	*Inulin and glycerol*	Rakicka et al. (2016)
Asparagus	High-fructose syrup	*K. marxianus*	
Y. lipolytica	Citric acid	*K. marxianus*	Liu et al. (2010)
Raw dahlia tuber	Ethanol	*K. marxianus*	Singh et al. (2007)
Jerusalem artichoke	Lactic acid	*A. niger* and *Lactobacillus* sp.	Ge et al. (2009)
Jerusalem artichoke	Single-cell oil	*Rhodotorula mucilaginosa*	Zhao et al. (2010)
Jerusalem	Sorbitol	*Kluyeveromyces*; *Saccharomyces cerevisiae*	Wei et al. (2001)
	Single-cell protein	*Y. lipolytica*	Chi et al. (2011)

sources have been tested for inulin, sucrose and glucose. Out of all of these carbon source tested, inulin hiked up inulinase activity (9.4 U/ml), followed by sucrose (6.7 U/ml) (Lu et al. 2014). At the same time same type of nitrogen source produced different effect on inulinase production. Accordingly, nitrogen source varies the production of inulinase with different species. There are organic and inorganic nitrogen sources described. Yeast extract and urea has been observed the best nitrogen sources. For *Bacillus smithii* and *Kluyveromyces marxianus*, $(NH_4)H_2PO_4$ and $(NH_4)2SO_4$ have been found the best nitrogen sources (Gao et al. 2009) (Yépez Silva-Santisteban et al. 2009). Further, sodium nitrate showed enhancement in the production when cultured with *Fusarium oxysporum*, whereas it has constrained the same when cultured with *Kluyveromyces fragilis* (Gupta et al. 1990).

The optimum temperature for inulinase production found to be 37 °C. Thermostability performs a vital role in industrial applications as it helps to prevent microbial contamination of the reactors (Kango and Jain 2011). Aeration and agitation has great significance in increasing the inulinase activity. By varying the culture volume, aeration can be tested. The more the culture volume, the lesser will be the enzyme activity (Yépez Silva-Santisteban and Maugeri Filho 2005). Agitation improved the inulinase production. At 180 rpm the inulinase activity was found to be maximum, but above 200 rpm it was supposed to reduce cell growth, hence inhibiting the process (Lu et al. 2014).

12.5 Industrial Advances

There have been a series of advances for utilizing inulinase activity on inulin for further production into various end level of products. High fructose syrup, ethanol, lactic acid and single-cell oil are some of the known series of high-end products that have produced utilizing various raw agricultural sources. Table 12.1 describes all of these series of products.

12.6 Future Work and Applications

Inulinase-significant issues at industrial scale are of great importance, but its extraction from various fungal sources is a difficult task. Presence of hyphae makes its harvesting very difficult. Searching and selecting an effective substrate for high production of inulinase, so that it can be further processed to bioethanol production (Miremadi and Shah 2012). Structural information has been available including 3D structures, but still a structure-function relationship need to be entrenched which can enhance molecular mechanism (Marina Holyavka 2016).

12.6.1 Health Benefits

Inulin is a rich source of fibres and improves digestion. Moreover it is beneficial for the intestinal bacteria, lowers cholesterol, prevents heart disease, boosts immune system and lowers blood sugar levels also. It also lifts bone health as mineral absorption is enhanced by inulinase. Inulin helps in measuring glomerular filtration rate which reveals the functioning of the kidney. GFR is defined as the volume of fluid filtered from the Bowman's capsule through glomerular capillaries per unit time.

12.6.2 Food Industry

It is a substitute for sucrose in many food items including bakery and confectionary products. Furthermore, it is used to produce high-fructose syrups which don't require insulin for its metabolism. Fructooligosaccharides (FOS) has been extensively used in food industry and further classified into prebiotic and post-biotic benefits. Inulin now has been extensively used in processed food items as it has extraordinary flexible features. It can be used to replace sucrose, fat and flour.

12.6.3 Bioprocessing Industry

Inulin can be used to process bioethanol, single-cell oil and single-cell protein, and can be an alternative for vegetables oils. Inulin that cannot be hydrolysed is directly

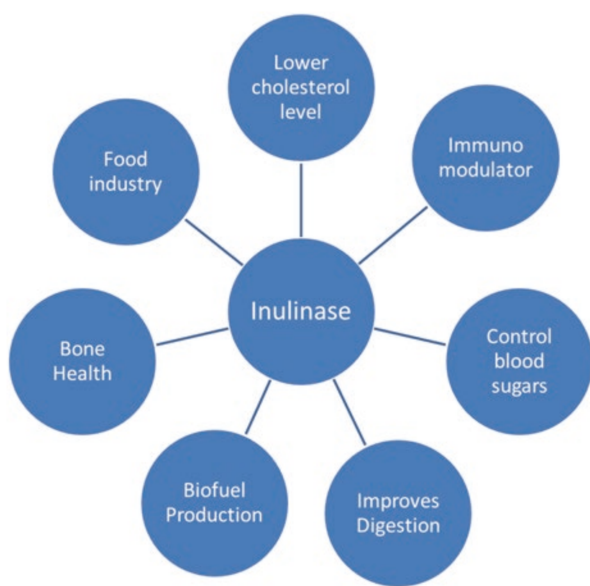

Fig. 12.4 Applications of inulinase enzyme in different areas

converted to ethanol by saccharification and by fermentation through which ethanol is produced and is used as fuels.

12.6.4 Other Chemical Production

Various different chemicals as lactic acid, 2, 3 butanediol and sugars like sorbitol and mannitol can be produced by inulinase. All these chemicals have vital roles in different fields like pharmaceuticals, cosmetics, food, medicine and chemical industries. This application makes inulinase an industrial friend. Figure 12.4 describes various fields for application of inulinases.

12.7 Conclusion

Inulin and inulin-containing products showed a promising industrial application. Ethanol has been produced by numerous fungal species and has been described in plentiful of research works. Many of the metabolic engineering works revealed the production of ethanol, through modification of metabolic pathways of bountiful of the fungal and yeast strains. Further fructose and fructooligosaccharides have emerged as vital additive in the food and pharmaceutical industries. Moreover, fructose found to be beneficial sweetener to sucrose as it has favourable outcome in reducing diabetic patients and elevated iron absorption in children. Fructose syrup was firstly obtained from corn starch, but nowadays both fructose and fructooligosaccharides are products of inulin. Optimized high temperature and thermostability

are the significant benchmarks to analyse. The inulinase can be distinguished based on the source from which it is obtained. The inulinases rarely show activity with sucrose and split fructans of the inulin type either endo-wise or exo-wise and produced a series of oligo-fructans or only fructose, respectively. Most of the inulinases are exo-enzymes that split fructose units from the fructose end of the inulin molecule. A few endo-enzymes have been reported to liberate oligo-fructosides as primary products of hydrolysis. Furthermore structural-metabolic relationship for the forthcoming investigation is a major challenge that can hike its applications in other sectors also.

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13 Plant Vaccines: An Overview

Gaurav Kumar, Loganathan Karthik,
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Abstract

Since the ancient times, microbial diseases remain to be one of the leading causes of mortality worldwide. Historical literature suggests the devastating effects of microbial diseases outbreaks on the socioeconomic of several countries. In such state, discovery and application of vaccines (microbial origin) gave new hope to the mankind to fight microbial infections in effective ways. Though being very effective, their production complexity, high cost, stability, and application methods remain to be the significant challenges in various countries. Recently, plant vaccines are attaining global attention owing to its numerous advantages over conventional vaccines including ease of production, cost-effectiveness, stability, and easy application (oral). Therefore, this chapter is an effort to summarize the various aspects of plant vaccine including its production, mode of action, and examples.

Keywords

Vaccines · Microbial diseases · Plant vaccines

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13.1 Introduction

Discovery of vaccine is certainly one of the most important medical inventions of the eighteenth century. In pre-vaccine era, microbial infections were major causes of mortality in humans and animals. Development and worldwide application of vaccines have significantly reduced the number of deaths caused by infectious diseases (Andre et al. 2008). Wide coverage of smallpox vaccine and oral polio vaccine (OPV) has helped in the elimination of smallpox and type 2 poliovirus globally (though types 1 and 3 polio are still reported). Combined measles, mumps, and rubella (MMR) vaccines have also reduced the death rate due to rubella and mumps in the vaccine coverage areas. Vaccines have a glorious past and bright future to represent them as an imperative candidate for future research; it all started in 1796, when Edward Jenner attempted vaccination for smallpox on an 8-year-old boy, James Phipps. He inoculated him with fluid collected from pustules obtained from a milkman suffering from cowpox. Subsequently, Phipps was inoculated with smallpox virus; surprisingly, he did not show any symptoms of full-blown smallpox infection. Later, Louis Pasteur observed that the attenuated strains of a pathogen can produce immunity in the host against the same organism. He named these attenuated strains as vaccine (Latin *vacca*, meaning cow).

Vaccine is a biological preparation that provides active immunity to the humans and animals. According to World Health Organization (WHO), a vaccine is any preparation intended to produce immunity to a disease by stimulating the production of antibodies (WHO 2018). A vaccine could possess purified microbial toxins, biomolecules, killed or attenuated microorganisms, and recombinant vectors. Any vaccine possesses two basic immunological characteristics: specificity and memory. In the twentieth century, remarkable development and understanding of technology provided significant progress in the development of vaccines. Currently, nearly 25 microbial infections can be prevented by vaccines (Table 13.1).

13.2 Type of Vaccines

In recent past, several types of vaccines have been developed. Theses vaccines vary in their production methods, stability, and mode of action. However, all vaccines are designed to trigger the immune response in the body.

13.2.1 Live Attenuated Vaccines

A live attenuated vaccine is a live microbial preparation in which microbes have been weakened by growing them on nonspecific host. Attenuated vaccine produces similar immune response as of wild strain, however doesn’t cause disease. It produces a strong immune response that often lasts lifelong.

Table 13.1 Vaccines available for human diseases

Virus	Type of vaccine	Vaccine	Disease
Influenza virus	Attenuated vaccine	Influenza vaccine	Influenza or flu
Measles virus	Attenuated vaccine	MMR, MMRV vaccine	Measles
Mumps virus	Attenuated vaccine	MMR, MMRV vaccine	Mumps
Rubella virus	Attenuated vaccine	MMR, MMRV vaccine	Rubella
Varicella zoster virus	Attenuated vaccine	MMRV vaccine	Chicken pox
Rotavirus	Attenuated vaccine	Rotavirus vaccine	Rotaviral gastroenteritis
Yellow fever virus	Attenuated vaccine	Yellow fever vaccine	Yellow fever
Mycobacterium tuberculosis	Attenuated vaccine	Bacillus Calmette-Guerin (BCG) vaccine	Tuberculosis
Variola virus	Attenuated vaccine	Smallpox vaccine	Smallpox
Salmonella typhi	Attenuated vaccine	Ty21a typhoid vaccine	Typhoid fever
Polio virus	Attenuated vaccine	Oral polio vaccine (OPV)	Polio
Polio virus	Inactivated vaccine	Inactivated polio vaccine (IPV)	Polio
Rabies virus	Inactivated vaccine	Rabies vaccine	Rabies
Japanese encephalitis virus	Inactivated vaccine	Japanese encephalitis vaccine	Japanese encephalitis
Bordetella pertussis	Inactivated vaccine	DPT	Whooping cough
Bordetella pertussis	Subunit vaccine	DTaP	Whooping cough
Hepatitis B virus	Subunit vaccines	Hepatitis B vaccine	Hepatitis B
Human papilloma virus	Subunit vaccines	Human papilloma virus vaccine	Cervical cancer, genital warts
Bacillus anthracis	Subunit vaccines	Anthrax vaccine	Anthrax
Salmonella typhi	Subunit vaccine	Vi capsular polysaccharide vaccine	Typhoid fever
Clostridium tetani	Toxiod	TT, DPT, DTaP, DT	Tetanus
Clostridium diphtheria	Toxiod	DPT, DTaP, DT	Diphtheria
Streptococcus pneumonia	Conjugate vaccine	Pneumococcal conjugate vaccine	Pneumococcal pneumonia

(continued)

Table 13.1 (continued)

Virus	Type of vaccine	Vaccine	Disease
Haemophilus influenzae type B	Conjugate vaccine	Hib vaccine	Pneumonia, acute meningitis
Neisseria meningitidis	Conjugate vaccine	Meningococcal vaccine	Meningococcal meningitis

13.2.2 Inactivated Vaccine

Inactivated vaccine is a killed microbial preparation in which microbes have been killed by chemical or physical treatment. These vaccines are safer and more stable than attenuated vaccine. An inactivated vaccine provides low immunity therefore is needed to be administered in multiple doses.

13.2.3 Subunit Vaccines

Subunit vaccines do not use the entire microorganism; in its place these vaccines use only the antigens that can induce the immune system effectively. These vaccines can be prepared by purifying the desired antigen from the pathogenic organism or by genetic engineering. A gene coding the vaccine protein of interest is inserted into a vector and subsequently expressed in a host system. These vaccines are safe and possess minimum side effects due to the use of a specific gene.

13.2.4 Toxoid

A disease caused by microbial toxins instead of microorganism itself can be cured by toxoid. A toxoid vaccine is an inactivated form of microbial toxins; the inactivation can be achieved by the treatment of toxin by chemical such as formaldehyde.

13.2.5 Conjugate Vaccines

Conjugate vaccines are a variation of subunit vaccines. This vaccine was developed to provide immunization against certain microorganisms that possess a polysaccharide layer around them. This layer protects the microorganism from the host's immune system. In conjugate vaccine, antigenic proteins are linked with microbial polysaccharide so that immune system can produce immunity against it. This polysaccharide attachment helps the immune system to defend the body by reacting with the polysaccharide coating of the pathogen.

13.2.6 DNA Vaccines

This is a very recent technology. In this technique, gene encoding for microbial antigens is directly introduced into host body. Then the microbial gene controls the host cell to produce the antigen protein of interest. Expression of microbial protein in host cells result in the specific immune response in host cell.

13.2.7 Recombinant Vector Vaccines

Recombinant vector vaccines are functionally similar to DNA vaccines, but attenuated virus or bacteria are used to introduce the microbial DNA into the host cells. Vector term is used for the attenuated virus or bacteria that used to carry the microbial DNA.

13.3 Plant Vaccines

Development of new techniques and better understanding of genetic engineering and plant biotechnology provide several opportunities to develop pharmaceutical products in plants. In the early 1990s, a novel approach to produce edible vaccine in plants emerged. Since then, many research groups have put a great effort for the development of technology for vaccine production in plants. Plant vaccines are a type of subunit vaccines and commonly called edible vaccines. Plant-derived vaccines are less toxic and cheaper than the conventional vaccines. The oral activity and heat stability of these vaccines make them an obvious choice for poor countries. Currently many plant-derived vaccines are available, and few of them are in clinical trials, the details and examples of which have been enlisted toward the end of the chapter.

13.4 Production of Plant Vaccines

Mason and Arntzen (1995) described detailed procedure for the production of plant vaccines. Plant vaccines are produced by molecular farming. It is an advanced biological technique used for the production of pharmaceutically beneficial proteins in plants. Plant vaccine is produced by inserting a microbial gene (encodes the microbial protein) into a plant. Expression of this microbial gene in plant cells will produce the protein that can be purified or consumed along with the plant tissue. Production of plant vaccines are illustrated in Fig. 13.1.

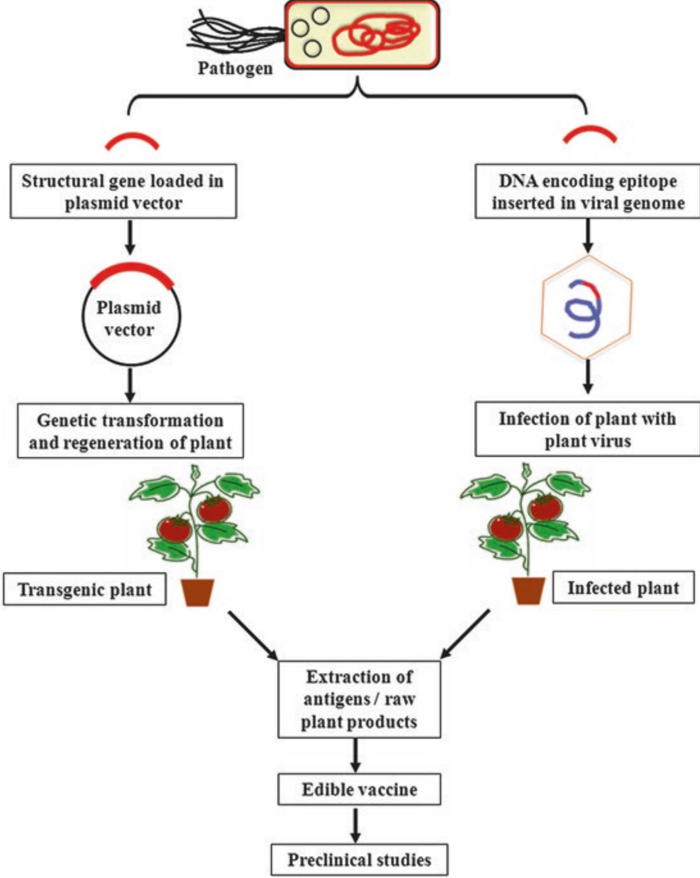

Fig. 13.1 Production of plant vaccine. (Adapted from Mason and Arntzen 1995)

13.4.1 Selection of the Gene

This is an important aspect of the plant vaccine production. The gene encoding the antigenic proteins that stimulate the immune system should be selected for the process. The requisitions of the gene include its ability to assemble in virus or plasmid, to survive in GIT, and also to trigger the oral and mucosal immune responses.

13.4.2 Selection of Plant

Selection of plant for the production of plant vaccine is based on many factors. A plant selected for the plant vaccine production should be easily available and must

be the one that can grow in the local region. The plant should be easy to transform and needs to express high quantity of protein. In addition, the plant must not contain any toxic compound in it. An edible plant is most preferred for both human and animal application. Currently, many plants have been used for the production of the plant vaccines such as tobacco, potato, tomato, banana, cereals, alfalfa, etc.

13.4.3 Insertion of the Gene in the Plant

The microbial gene can be transferred in plants by two methods. They are as followed:

13.4.3.1 Stable Genomic Interaction

The structural gene (antigenic gene) is loaded on a plant transformation vector. The vector is introduced into plant cells using *Agrobacterium tumefaciens* chromosomal integration or by microprojectile bombardment techniques.

13.4.3.2 Transient Expression Using Viral Vectors

The microbial DNA encoding for the epitope is inserted in a plant pathogenic virus. This genetically engineered virus infects the plant cells and starts replication there. Along with viral genes the microbial genes get expressed and produce protein that gets accumulated inside these cells.

13.4.4 Harvesting and Use of the Vaccine

Expression of microbial protein occurs in various parts of the plant. The plant tissue can be consumed directly or after cooking. Alternately, the protein can be purified by downstream processing and subsequently used in developing tablets, capsules, and other pharmaceutical products. Generally, plant vaccines are considered safe; however, it is important to check the potential and toxicity of the product in animal models before being used commercially.

13.5 Mode of Action

Earlier many researchers have summarized the mechanism of plant vaccines. Mishra and coworkers explained the mode of action in detail (Mishra et al. 2008). A plant vaccine administered orally stimulates the mucosal and humoral immune system of the host body. Mucosal immune system is the primary defense system and an appropriate site for vaccination. After the administration of plant vaccine, it stimulates the immune system in a systemic manner (Fig. 13.2).

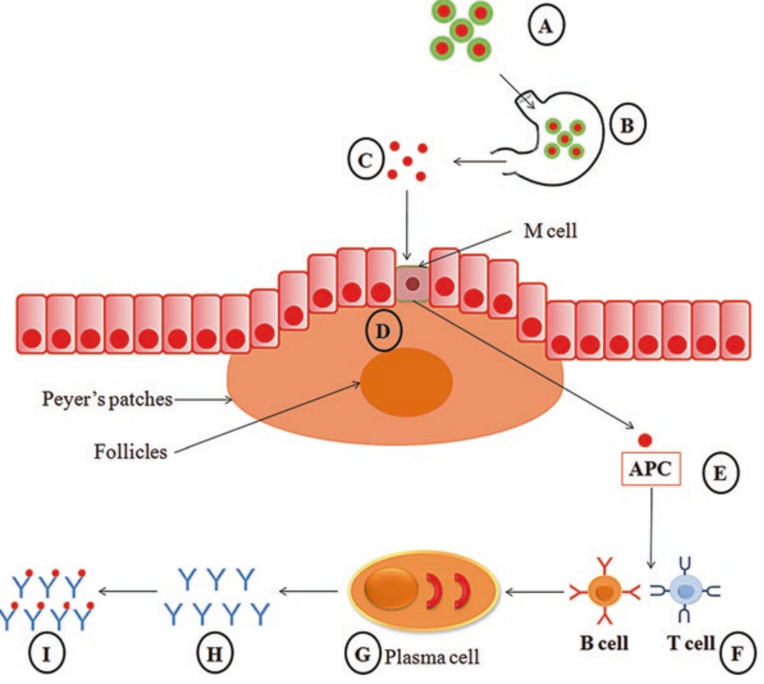

Fig. 13.2 Mode of action of plant vaccine. (Adapted from Mishra et al. 2008)
Here, (A) plant vaccine; (B) plant vaccine inside the GIT; (C) release of the antigen in the intestine; (D) absorbed of antigen by M cells; (E) M cells pass the antigens to antigen-presenting cells (APC); (F) activation of T and B lymphocytes; (G) differentiated of B cells into plasma cells; (H) production of IgA antibody; (I) neutralization of antigen

13.5.1 Entry of the Antigen

Antigen present in the plant cell can be consumed in the form of raw plant products such as fruits and vegetables. The tough outer cell wall protects the antigen from degradation by gastric enzymes and breaks to release of the antigen in the intestine.

13.5.2 Absorption of Antigen

Free antigen present in the intestine are absorbed by M cells (or microfold cells are found in the follicle-associated epithelium of the Peyer's patch) in the intestinal lining that are present over the Peyer's patches (in the ileum) and the gut-associated lymphoid tissue (GALT).

13.5.3 Immune Response

M cells pass the antigens to the macrophages and other antigen-presenting cells which further present the antigens to local T and B lymphocytes. These activated B cells migrate toward mucosal-associated lymphoid tissue (MALT). B lymphocytes differentiate into plasma cells and synthesize IgA antibodies at mucosal surfaces that neutralize the antigens in the intestine. In addition, few antigens taken up by the intestinal dendritic cells (DCs) that further induces the production of IgG antibodies, imparting systemic immunity.

13.6 Current Status of Plant Vaccines Research

Microbial infections are the leading cause of disease in poor and developing countries. Lack of prophylactic measures and treatment makes it difficult to control the microbial infections. In the last two decades, emergence of plant-derived vaccines has extended a hope for the development of safer, cheaper, and edible vaccine. In the recent past, various plants having vaccines have been prepared and tested. Some of them with respect to organism/disease, vaccine protein and the plant system for the expression of gene are being summarized henceforth.

13.6.1 Plant Vaccines Against Bacterial Infections

Bacterial diseases continue to pose a major threat to human health. Pathogenic bacteria cause a variety of infections such as tetanus, typhoid fever, diphtheria, syphilis, cholera, food-borne illness, leprosy, and tuberculosis. Tuberculosis ranks second among the world's leading causes of death worldwide. In year 2012, 8.6 million cases and 1.3 million deaths were reported due to tuberculosis (WHO 2013). *Streptococcus* (group B *Streptococcus*) is another frequent cause of life-threatening infection during the first 2 months of life. Food- and water-borne bacteria such as *Salmonella* and *Campylobacter* are responsible for a recent dramatic increase in diarrheal disease.

The discovery of new organisms and new strains of many familiar bacteria presents a sturdy challenge to the researchers toward development of microbial control measures. There is a large and fast-growing list of target protein/peptide from microbial pathogens that have been expressed by plants. Some of them are listed in Table 13.2.

13.6.2 Vaccines Against Viral Infections

Viruses cause various dreadful diseases in human and animals. Most of the epidemic diseases are caused by viruses because of their ability to spread fast. Viral infections are difficult to treat, because they grow inside the host cell and quickly

Table 13.2 Vaccines against bacterial diseases

Pathogen	Target protein/peptide	Plant	References
Enterotoxigenic *Escherichia coli*	Heat labile enterotoxin B subunit (LT-B)	Tobacco	Haq et al. (1995)
	LT-B	Potato	Haq et al. (1995)
	LT-B	Tobacco	Kang et al. (2003)
	LT-B	Maize kernels	Chikwamba et al. (2003)
	LT-B	Soybean	Moravec et al. (2007)
Vibrio cholera	Cholera toxin B-subunit (CT-B)	Potato	Arakawa et al. (1998)
	CT-B	Tobacco	Daniell et al. (2001)
	CT-B	Tomato	Jiang et al. (2007)
	CT-B	Rice	Nochi et al. (2007)
Clostridium tetani	TetC	Tobacco	Tregoning et al. (2003)
Yersinia pestis	F1 and LcrV antigens	Tobacco leaf	Mett et al. (2007)
Borrelia burgdorferi	OspA, OspA-T	Tobacco	Glenz et al. (2006)
Staphylococcus aureus	D2 peptide of fibronectin-binding protein FnBP	Cowpea leaf and tobacco leaf	Brennan et al. (1999a, b)
Pseudomonas aeruginosa	Peptides of outer membrane protein F	Cowpea leaf and tobacco leaf	Gilleland et al. (2000) and Staczek et al. (2000)
Mycobacterium tuberculosis	LT-B and early secretory antigen	*Arabidopsis thaliana*	Rigano et al. (2004)

adapt to new environment by making changes in the structure. Human immunodeficiency virus, hepatitis virus, rabies virus, variola virus, polio virus, and Japanese encephalitis virus are some of the most common viral pathogens. These viral pathogens cause severe infections in humans and resulted in several death worldwide. Application of conventional vaccines has controlled the death rate of viral diseases; however, development of plant vaccines could possibly provide a support to the conventional vaccines. Various viral proteins are expressed in plants for the preparation of edible vaccine; some of them are summarized in Table 13.3.

13.6.3 Plant Vaccines Against Parasites

In tropical and subtropical regions of the world, parasitic infections are leading causes of diseases and subsequent deaths. Malaria, lymphatic filariasis, and toxoplasmosis are the most common and severe parasitic diseases. Malaria is an endemic disease occurring in almost 108 countries of the world and kills a large number of people every year (WHO 2016). Nearly 30 million people in the United States are affected with toxoplasmosis according to the report of Centers for Disease Control and Prevention (CDC) (CDC 2017). About 120 million people are infected with lymphatic filariasis worldwide, 65% of them are present in Southeast Asia region

Table 13.3 Plant vaccines for viral pathogens

Viral pathogen	Target protein/peptide	Plant	References
Hepatitis B virus	Surface	Tobacco	Mason et al. (1992)
	Surface	Potato	Richter et al. (2000)
	Surface	Carrot	Joung et al. (2004)
	Surface	Banana	Kumar et al. (2006)
	Surface	Tomato	Li et al. (2011)
HIV type 1 virus	V3 loop of gp120 protein	Tobacco leaf	Yusibov et al. (1997)
	Peptide of V3 loop of gp120 protein	Tobacco leaf	Joelson et al. (1997)
	Nucleocapsid protein p24	Tobacco leaf	Zhang et al. (2002)
	Tat protein	Spinach	Karasev et al. (2005)
Rotavirus	(VP6) protein	Alfalfa	Dong et al. (2005)
Variola virus	B5 antigenic domain (pB5)	Tobacco and collard leaf	Golovkin et al. (2007)
Japanese encephalitis virus	Envelope protein	Rice	Wang et al. (2009)
Pathogenic avian influenza virus	H5N1	Tobacco	Shoji et al. (2009)
Lyssavirus	Glycoprotein	Maize	Loza-Rubio et al. (2008)
Human papilloma virus type 16	E7 oncoprotein	Tobacco leaf	Franconi et al. (2002)
Rabies virus	Glycoprotein	Tomato leaf and fruit	McGarvey et al. (1995)
	Glycoprotein and nucleoprotein	Tobacco and spinach leaf	Yusibov et al. (2002)

Table 13.4 Plant vaccines against parasites

Parasites	Target protein/peptide	Plant	References
Plasmodium yoelii	Merozoite surface protein (PyMSP4/5)	Tobacco	Wang et al. (2008)
P. falciparum	Peptides of circumsporozoite protein	Tobacco	Turpen et al. (1995)
Entamoeba histolytica	LecA, a surface antigen	Tobacco	Chebolu and Daniell (2007)
Toxoplasma gondii	Surface antigen 1 (SAG1)	Tobacco	Clemente et al. (2005)

(CDC 2013). Development of edible vaccines to prevent the parasitic infection could provide an alternative to the existing prophylaxis and treatment methods. At present, some parasitic proteins are expressed in plants for the preparation of edible vaccine, few of which are summarized in Table 13.4.

Table 13.5 Plant vaccines against autoimmune disease

Autoimmune disease	Target protein/peptide	Plant	References
Diabetes-associated autoantigen glutamic acid decarboxylase	GAD linked to the innocuous B subunit of the *V. cholerae* toxin	Tobacco and potato plants	Ma and Jevnikar (1999)
Arthritis	Antitumor necrosis	Carrot cell	http://www.molecularfarming.com
	Factor		
Diabetes	Insulin	Safflower	http://www.molecularfarming.com

13.6.4 Plant Vaccines Against Autoimmune Disorders

Autoimmune disorders occur when the body produces an inappropriate immune response against self. This immune response causes inflammation and damages the tissue leading to conditions like arthritis, multiple sclerosis, type I diabetes, myasthenia gravis, etc.

Most of the autoimmune disorders as of now lack a successful treatment, and therefore the imminent need is to develop a cure. Some researchers have reported the expression of protein into plant for developing vaccine against arthritis and diabetes (Table 13.5). These proteins can be used to develop vaccines for the control of autoimmune disorders.

13.7 Advantage of Plant Vaccines

Plant vaccines possess several advantages over conventional vaccines. They are highly cost effective with regard to production, storage and transportation cost. These vaccines are produced in transgenic plants, and their production and processing require simple agricultural techniques, thereby reducing expenditure of equipment and technology. Transgenic plants provide higher yield of antigenic proteins compared to mammalian cell culture; however, production varies within the plant species. Although purification of antigenic protein is the most expensive step in the entire production process, yet it is easier than that in bacterial or mammalian cell. Plant vaccines are stable at room temperature thereby nullifying the need for cold chain storage system. For example, transgenic cereal grains containing antigenic proteins can be stored at room temperature for almost a year. Vaccines from plants are usually delivered to by oral route consequently decreasing the necessity of a medical professional to deliver the same. Oral vaccine reduces the cost of medical equipment as well as protects the environment from the pollution caused by unsafe disposal of used syringe and needle required during the administration of conventional vaccines. Most importantly, oral vaccines from transgenic plants reduce the chances of cross infections which may occur because of the use of contaminated needles that have been reported to spread infections such as HIV, hepatitis, malaria, brucellosis, syphilis, toxoplasmosis, etc.

13.8 Disadvantage of Plant Vaccines

As presence of two sides to the same coin is indispensable; disadvantages are inevitable. Amidst the several advantages, plant vaccines possess few drawbacks which need to be rectified in the future. Plant vaccines are not convenient for infants. Their dose may vary based on age and size of plant product (fruit and tubers) since diversity in age and size of plant product may express different levels of protein. Possibility of contamination of plant products or vaccines with mycotoxins, pesticides, and insecticides is capable of causing side effects in host. Presence of certain toxic phytochemicals in plant could produce adverse reaction in host body. In addition, mixing of transgenic plants with their normal counterparts can cause overdose of the vaccine in the consumers.

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Microbial Biosurfactants: Future Active Food Ingredients

14

Vikrant Sharma and Deepansh Sharma

Abstract

Microbial surfactants are a structurally diverse group of surface-active molecules produced by microorganisms. The rising environmental apprehension about synthetic surfactants elicits awareness to microbial surfactants vitally because of low toxicity, stability to extreme environmental conditions, and biodegradability. Biosurfactants are mostly used in environmental removal of pollutants; however, biosurfactant also exhibits significant utility in various prospects of food processing sector. Emulsion formation, oil-water stabilization, and anti-biofilm, antiadhesive, and antimicrobial potential are characteristics of microbial surfactants, which might be considered as an active ingredient in food processing and formulation. Bearing in mind the community and scientific backgrounds, utilization of microbial surfactants, which are eco-friendly and significantly important, has turned out to be vital for food-related applications.

Keywords

Biosurfactant · Food industries · Antiadhesive · Antimicrobial · Emulsifiers

14.1 Introduction

Biosurfactants are amphiphilic molecules comprising hydrophobic and hydrophilic fractions that are put together to gather at interface, reducing the surface and interfacial tensions, and forming emulsions (Koglin et al. 2010; Lima et al. 2011; Xu

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et al. 2011; Desai and Banat 1997; Saharan et al. 2011, 2015; Sharma and Singh Saharan 2014). Biosurfactants engrossed consideration as hydrocarbon removal substances, but the attention in biosurfactants has been increasing significantly in the last decades as substitute to synthetic surfactants particularly in food and pharmaceutical industries (Banat et al. 2000; Nitschke and Costa 2007and Thavasi et al. 2008). Biosurfactants are of eco-friendly behavior having low toxicity, as biosurfactants are certainly biodegradable with distinct structures which offer significant potential that conventional chemical surfactants may lack (Mohan et al. 2006; Flasz et al. 1998). Most of the research on biosurfactants till now mainly focused toward the removal of pollutants from contaminated sites (Mulligan 2005; Banat et al. 2010). However, biosurfactant displays significant potential for the food processing particularly as emulsifiers and solubilizers (Desai and Banat 1997 Banat et al. 2000) and antiadhesive and antimicrobial agents (Stadler et al. 2014; Gudiña et al. 2011; Singh and Cameotra 2004). Microbial surfactants are natural choice over conventional surfactants and are favorite over chemical surfactants for the huge range of industrial utilities, pharmaceuticals, cosmetics formulations, and food and feed sector (Makkar et al. 2011). There is a increaseing awareness to drop the use of synthetic surfactants and explore the possibilities of the biosurfactants derived from the GRAS microorganisms (Shepherd et al. 1995). In spite of the benefits governed by microbial surfactants, only some reports are existing regarding their application in food processing. The current chapter argues the potential applications of biosurfactants for food and food-associated industries.

14.2 Classification of Biosurfactants

Microbial surfactants have been categorized on the basis of the nature of their moieties such as polar and functional groups, while biosurfactants are classified mainly on the basis of composition and type of microorganisms involved. Biosurfactants can be categorized in two major categories, mainly low-molecular-weight biosurfactants, which competently lower surface and interfacial tension, and high-molecular-weight biosurfactants, which are mainly emulsion or stabilizing agents. Low-molecular-weight biosurfactants comprise glycolipids, lipopeptides, and phospholipids, while high-molecular-weight comprise polymeric and particulate surface-active agents. Major biosurfactants on the basis of total charge may be anionic or neutral, and the hydrophobic moiety is mainly composed of long-chain fatty acids or fatty acid derivatives, while the hydrophilic moiety mainly comprises carbohydrate, amino acid, phosphate, or cyclic peptide. Glycolipids are the major catagory of low molecular mass biosurfactants obtianed form the microorganisms. Glycolipids mainly comprise of long-chain aliphatic acids or hydroxyaliphatic acids. Between the glycolipids, the most known biosurfactants are rhamnolipids, trehalolipids, and sophorolipids. Rhamnolipids (RL) in which one or two molecules of rhamnose are associated with the chain of β-hydroxydecanoic acid. The production of rhamnose-associated glycolipids was first reported in *Pseudomonas aeruginosa* (Jarvis and Johnson 1949). They have been intensively inspected and extensively reviewed (Ochsner et al. 1996; Nitschke et al. 2005; Soberón-Chávez and Maier 2011).

Several structural types of microbial trehalolipid biosurfactants have been reported (Li et al. 1984. Sophorolipids are mainly obtained from yeast-like *Torulopsis bombicola* (Cooper and Paddock 1984), composed of a dimeric carbohydrate sophorose along with a long-chain hydroxy fatty acid. Sophorolipids are the mixture of at least six to nine different hydrophobic sophorosides (Hommel et al. 1987; Shao et al. 2012; Price et al. 2012; Maddikeri et al. 2015).

14.3 Biosurfactants in Food Formulation and Processing

Microbial surfactants are biocompatible, biodegradable, and nontoxic compounds with exceptional properties that display a variety of significant properties for the food sector (Banat et al. 2000; Sharma et al. 2015; Campos et al. 2013; Muthusamy et al. 2008) anti adhesive, and antimicrobial agents (Gudiña et al. 2010; Sharma et al. 2015; Singh and Cameotra 2004) and food additives (Nitschke et al. 2007) potential has bring about in an increased attention in finding novel strains for biosurfactant production suitable for used in new and innovative formulations in the food sector.

14.3.1 Biosurfactants as Food Stabilizer

Biosurfactants are adjusting the consistency in bakery products, creams, and ice cream productions. They are also consumed as fat stabilizer and anti-spattering substances in cooking of oil and fats (Kosaric 2001; Magalhães and Nitschke 2013). Neta et al. (2012) reported the synthesis of surface-active stabilizers of coconut milk emulsions by enzymatic synthesis of sugar esters. Sugar esters displayed the significant surfactant properties (biosurfactants), i.e., capable of reducing the surface tension, and promote the emulsification. Quality and shelf life of bread were improved by accumulation of biosurfactant obtained from *Bacillus subtilis*. The influence of *Bacillus subtilis*-derived biosurfactant has been evaluated in comparison to a commercial surfactant. For the emulsification, lecithin and its derivatives are used in the industries. Biosurfactants showed a significant anti-staling effect in bread crumb texture evolution during storage. Additionally, biosurfactant incorporation reduced the chances or delayed the chances of microbial contamination (Mnif et al. 2012). Biosurfactant incorporation improves the consistency and modifies rheological properties and shelf life of products. Biosurfactants can also improve the texture of fat-based formulations by controlling the agglomeration of fat globules (Makkar et al. 2011). L-Rhamnose is previously known for various food applications such as precursor of flavor development like furaneol which is derived by hydrolysis of rhamnolipid derived from *P. aeruginosa* (Linhardt et al. 1989). Also, the biosurfactant derived from *Streptococcus* spp. can be used to control the growth of *S. thermophilus* in the pasteurizer, which can lead to the fouling of the product produced.

14.3.2 Role of Biosurfactants in Emulsion Forming

An emulsion is a mixture of two or more liquids that are generally immiscible (non-mixable or unblendable). The incorporation of emulsifiers develops the texture and mouthfeel of dairy products (Rosenberg and Ron 1999). Dairy products retain a minimal stability, which may be improved by certain food additives such as biosurfactants (Velikonja and Kosaric 1993). This property is particularly advantageous for making oil/water emulsions for food formulations. Mostly, bioemulsifiers are microbial products with a hydrophobic moiety and a hydrophilic moiety. A number of biosurfactants are obtined from the microorganisms, out of which glycolipids, e.g., rhamnolipid, lipopetides, emulsan and the polysaccharide-protein complexes are the most studied biosurfactants (Cirigliano and Carman 1984, 1985; Kosaric and Sukan 1993). High-molecular-mass biosurfactants are very significant for emulsification properties as compared to the low-molecular-weight biosurfactants. Biosurfactants obtained from *Torulopsis bombicola* have significantly reduced the surface and interfacial tension but were not found to be good emulsifiers (Cooper and Paddock 1984). In dairy products, the addition of emulsifiers improves the texture and creaminess especially in soft cheese and cream-based preparations. Biosurfactants obtained from the *C. utilis* showed low viscosity and had high carbohydrate content of above 80%. Experimental observations showed the bioemulsifier obtained had potential application in formulation of salad cream (Shepherd et al. 1995). Some efforts have been made to assess emulsion-forming potential of biosurfactants with oils and fats consumed in food industry and formulations. Soybean oil and coconut fat were reported to form stable emulsions with the lipopeptide obtained from *B. subtilis*, signifying the lipopeptide's role as an emulsifying agent (Nitschke and Pastore 2006). Corn oil and water emulsion obtained with a mannoprotein derived from *Kluyveromyces marxianus* was observed and found to have stable emulsion for 3 months which advocated their role as bioemulsifier in food processing (Lukondeh et al. 2003). Emulsifying agents are successfully used as dressing formulations such as *Candida utilis*-derived carbohydrate-rich biosurfactants in salad (Shepherd et al. 1995). The biosurfactants obtained from generally recognized as safe (GRAS) microbes are already present in many food applications such as mannoprotein derived from *Saccharomyces cerevisiae* that has shown emulsion-forming properties with various cream-based formulations on various pH ranges. Various other yeast species have been reported for biosurfactant production and could be of huge interest in food processing such as *Candida valida*, *Candida utilis*, and *Rhodotorula graminis* with better stabilizing activity.

14.3.3 Biosurfactants as Food Ingredients/Additives

Food additives are generally defined as constituents without nutritional value which are generally used to transform physical, chemical, biological, and sensory characteristics during food processing. A vital principle of additive applications in food is safety evaluation; therefore, before approval for use, an additive essentially fulfills

the toxicological evaluation (Brasil, 2002). Biosurfactants can be used for controlling the agglomeration of fat, develop texture and increase shelf life of food, and transform rheological characteristics with better consistency and texture of oil- and fat-based formulations (Kachholz and Schlingmann 1987). In bakery products, microbial surfactants solubilize flavor oils and control their consistency (Kosaric 2001). Rhamnolipids have been reported for the application as food ingredients in dough improvement and stability. Rhamnolipids also improve properties of butter cream (Van Haesendonck and Vanzeveren 2004). Bioemulsifier isolated from *Enterobacter cloacae* was described as a potential viscosity enhancement agent of interest in food industry (Iyer et al. 2006). Biosurfactants also showed antioxidant properties and could be potent antioxidant source in food preparations. Mannosylerythritol lipids were reported for their antioxidant potential to scavenge superoxides (Takahashi et al. 2011). Mannosylerythritol lipids have maximal antioxidant effects in cells and advocate the potential use in cosmetics formulations. Biosurfactants obtained from the *B. subtilis* RW-I also exhibit the potential antioxidant properties to scavenge free radicals and recommend the role of biosurfactants as novel antioxidants as compared to the natural antioxidants.

14.3.4 Antimicrobial Potential

Biosurfactants with significant antimicrobial action to control the pathogenic bacterial and fungal diseases have been documented in previous years (Gudina et al. 2010; Sharma and Singh Saharan 2014; Banat et al. 2010). A lipopeptide (iturin) obtained from the *B. subtilis* exhibits potent antifungal potential (Besson et al. 1976). Significant decline of mycoflora in various grains of corn, peanuts, and cottonseeds was observed at concentration ranging from 50 to 100 ppm (Klich et al. 1994). A rhamnolipid derived from *P. aeruginosa* showed inhibitory activity against the various food spoilage microorganisms at low concentration of biosurfactant (Abalos et al. 2001). Glycolipids type of biosurfactants exhibits significant antifungal potential such as sophorolipids and rhamnolipids. Sophorolipids and rhamnolipids were found as potential antifungal agents against mycelial growth of *Phytophthora* sp. and *Pythium* sp. (Yoo et al. 2005). Other glycolipids such as mannosylerythritol lipid, derived from *Candida antarctica*, exhibit effective antimicrobial properties more specifically against Gram-positive bacterial pathogens (Kitamoto et al. 1993). Biosurfactants isolated from the *Lactobacillus paracasei* showed antimicrobial properties and antiadhesive properties against various food pathogens in different degree of inhibition (Gudina et al. 2010). The results obtained suggest the possible role of biosurfactant against various food pathogens as an alternative antimicrobial agent. As evident from literature thal all the *Lactobacilli* are of GRAS status and could be used as a food additive and emulsifying agent in food formulations. Most of the highly studied biosurfactants were produced from the *Bacillus* and *Pseudomonas* genera, but their opportunistic pathogenicity is always objectionable. Biosurfactants with combination of other food preservatives showed potent inhibition against food spoilage microorganisms. Antimicrobial activity of rhamnolipids against *Listeria*

monocytogenes and their synergistic interaction with nisin established the fact of combined preservative strategies (Magalhães and Nitschke 2013).

The antimicrobial potential has also being checked on the pathogens isolated from clinical samples (Dhouha Ghribi et al. 2012). The test was done by agar spot method described by Paik et al. (1997), and the results were clearly indicating that, with the increasing concentration of the biosurfactant, the antimicrobial activity also increases.

The antimicrobial potential, concomitant with the significant surface-reducing properties, high biodegradability, and low toxicity of biosurfactants, provides the opportunity to be a vital choice for combating against *L. monocytogenes* with a specific target application area in food industry. The emergence of antibiotic resistance among the foodborne pathogens, collective with the continuous demand of "natural" additives, encourages the search for next-generation bio-preservatives.

14.3.5 Antiadhesive Potential

Biofilm development can be defined as a survival strategy by a group of bacteria that have colonized along with the production of various extracellular metabolites produced at the surface which ultimately results in matrix formation (Hood and Zottola 1995). Initial steps of the biofilm formation involve adherence which involves interaction of different factors such as microorganism species, hydrophobicity of surface and charge of surface, environmental conditions, and most importantly the potential of the microorganisms to produce extracellular molecules which leads to matrix formation (Zottola 1994). The biofilm formation in food industry and processing are possible sources of contamination in industries. Prevention and control of biofilm in food processing and surfaces is a critical step in ensuring safe and quality products to the consumers (Hood and Zottola 1995). The role of biosurfactants in combating microbial adhesion, biofilm formation, and removal from surfaces has been observed in previous years. Biosurfactants derived from *Streptococcus thermophilus* have been observed to combat biofouling of heat exchanger plates in dairy industry (Busscher et al. 1996).

The preconditioning of heat exchanger surface with biosurfactants advocates the use of biosurfactants as a new approach to reduce adhesion. The biosurfactants obtained from human microflora and of probiotic origin are also very encouraging (Saharan et al. 2011; Sharma and Singh Saharan 2014; Sharma et al. 2015; Singh and Cameotra 2004). The biofilm formation by *Salmonella typhimurium*, *Salmonella enterica, E. coli*, and *Proteus mirabilis* has been controlled by biosurfactants (Mireles et al. 2001). The growth of various foodborne pathogens and biofilm-forming bacteria such as *Bacillus cereus* and *Listeria monocytogenes* has been reduced to a significant level by biosurfactants obtained from the *Lactobacillus helveticus* MRTL 9 (Sharma and Singh Saharan 2014).

Meylheuc et al. (2001) reported the control of biofilm form by *L. monocytogenes* in food industry by biosurfactants produced by *L. helveticus*. The stainless steel and

PTFE surfaces were preconditioned with a biosurfactant derived from *Pseudomonas fluorescens* which prevents the adhesion of pathogen to 90%. De Araujo et al. (2011) also observed similar effects while working with polystyrene surfaces. The pre-coating of surface by rhamnolipid and surfactin prevents the *Listeria monocytogenes* adhesion to the surface. Most strains of *L. monocytogenes* are proficient to cultivate antimicrobial-resistant biofilms on a range of food processing surfaces like stainless steel, PET, PTFE, PVC, and glass (Chae and Schraft 2000). Biosurfactants have been considered as promising versatile biomolecules, which display emulsifying, anti-biofilm, and antimicrobial potential at the same time and are subsequently appropriate for various food applications (Banat et al. 2010; Nitschke and Costa 2007). So, biosurfactants could be used as food additives directly to improve the stability of oil-based food preparation or, secondarily, as green cleaning formulations with detergency property to clean food processing surfaces (Freire et al. 2009).

In view of the interesting potential displayed by microbial surfactants, we can think of their future consumption as multipurpose molecules, with significant emulsification, anti-biofilm, and antimicrobial potential appropriate for various food applications. Food processing industries and research are still not using biosurfactants in direct applications on a commercial scale due to various regulating guidelines and issues. But the rising number of patents and technologies related to the biosurfactants advocating their use in food formulation, cosmetics ingredients, and nutraceutical formulations (Table 14.1) (Shete et al. 2006), signifying the rising attention in using biosurfactants.

Table 14.1 Recent patents trend on biosurfactant in the food sector

S. no.	Patent particulars	Application	Inventors
1	Microbial surfactant as active ingredient	Drugs, drinks, and foods	Suzuki et al. (2011)
2	Biosurfactants for blocking the interaction of a pathogen with a collagen receptor	Food preparation trays to block or inhibit pathogens from binding to the polymers or materials	Howard et al. (2004)
3	Cleaning fruits and vegetables; mixture of alpha hydroxy fruit acids; sodium lauryl sulfate; sophorose lipid biosurfactant	Germicidal and are sufficient to execute 100% population of *E. coli*, *Salmonella*, and *Shigella*	Pierce and Heilman (2001)
4	Formulations combining ramoplanin and rhamnolipids for combating bacterial infection	Control of *Enterococcus*, *Clostridium difficile*, or multidrug-resistant *Clostridium difficile*	Yin (2014)
5	Cyclic lipopeptides for use as taste modulators	Food, beverages, medicinal products, and cosmetics and contain preferably mono-, di-, or oligosaccharides as sweeteners	Krohn and Zinke (2011)

(continued)

Table 14.1 (continued)

S. no.	Patent particulars	Application	Inventors
6	Rhamnolipid compositions and related methods of use	Therapeutic agent	Gandhi et al. (2007)
7	Long-chain glycolipids useful to avoid perishing or microbial contamination of materials	Preservative or antimicrobial properties	Stadler et al. (2014)
8	A cleaning formulation of 0.01–99.9% of rhamnolipid for food preparation; toys, medical equipment; protective coatings; nontoxic	Inhibits the development of bacterial and fungal pathogens	DeSanto (2011)
9	Sophorose lipids	Cosmetics and pharmaceutical products	Lang et al. (2002)

14.4 Future Trends and Constraints

Biosurfactants show various potential characteristics which could be valuable in various food-related applications due to their antiadhesive nature which has engrossed openness as a new approach to prevent and disrupt the biofilm formation in food industries. Inadequate structural information, toxicity assesment with expensive production and maintenance costs appears to be the key cause for the restricted consumptions of biosurfactants in food sector. Development of inexpensive agro-industrial substrate for mass production of biosurfactant could be a major milestone to the near future. Further maximal production and low purification cost with exceptionally significant properties would increase the acceptance of these microbial products in industrial corridors. Meanwhile better policies and clinical trials for the biosurfactants based formulations, require attention of government agencies to draft the regulation related to their use. Biosurfactants produced by GRAS microbes such as *Lactobacilli* and food-associated yeasts might be of huge potential for food processing. Furthermore, tailor-made or enzymatic synthesis of glycolipids would reduce the cost of the purification and production yield. With a target to build a defensible society in promise with the environment, that the introduction of green chemicals and constituents in food industry is one of our utmost goal.

14.5 Conclusions

The search of novel microbial surfactants appropriate for food processing has been gradually increasing obsessed by industries looking to decrease the expectations on plant-derived emulsifiers. High production and recovery costs and complications to

produce sufficient quantities of biosurfactants hampered acceptance by industries. Using inexpensive substrates, strain improvements, optimization of process parameters, production, and downstream processing to increase yields can bring the required breakthrough. Through dedicated cost-effective applications in the food sector and investigations which lead to establish toxicity, biosurfactants would be the molecules of the future.

Conflict of Interest The author declares that there is no conflict of interest.

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Part III

Microorganisms as Future Tools

Microbial Spores: Concepts and Industrial Applications

15

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Abstract

Several microorganisms like bacteria, fungi, yeast, algae, actinomycetes and protozoa are well known for their ability to form spores. Spores have inherently distinct life cycle as compared to vegetative cells making them able to resist various unfavourable environmental conditions like extreme temperature, radiations, desiccation, toxic chemicals, etc. Keeping in view their vast potential, the use of spores has led to several breakthrough researches in order to develop a large number of spore-based products in the fields of biosensing, biocontrol, biofertilizers, biocatalysis, biosorption, biopolymers, biological warfare, medicine, probiotics and surface display. Most of these products are now available commercially and thus indicate the indispensable potential of microbial spores. The current chapter gives information about the concept of microbial spores and its potential industrial applications in aforementioned fields. Furthermore, attention has also been paid to the current status, associated challenges and future perspectives for spore-based technologies.

Keywords

Spores · Biosensing · Biocontrol · Probiotics · Applications

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15.1 Introduction

Microbial spores are robust and metabolically dormant structures that are produced by a process of sporulation to prevail over harsh and unfavourable climatic conditions of starvation and stress. They are formed by microorganisms belonging to different groups such as bacteria, fungi, actinomycetes, algae, etc. Spores are of great significance. The use of spores on one hand opens the door to a number of useful applications in various fields, but at the same time, they have also become the major cause for various types of food spoilages and food-borne diseases (Setlow 2006; Setlow and Johnson 2007; Coleman et al. 2010).

Physiological behaviour of spores that occurs as a part of their life cycle holds great promise for immense industrial applications. It is well known that the life of spores revolves around two phases, the dormant and the metabolically active vegetative state, and perhaps this exclusive biphasic phenomenon of spores could have great potential to form the basis of development of various techniques ranging from biosensing and biocontrol to probiotics and medicine. Development of these techniques based on the use of spores takes the advantage of the process of germination by examining the physiological changes occurring upon addition of spore-specific dormancy-breaking signals (Yung 2008). Induction of germination in spores by exposure to specific germinant converts them into vegetative cells which are metabolically very active and fragile. Conversion from dormant to vegetative forms takes place when spores sense favourable environmental conditions.

Taking advantage of the spore's unique resistance properties and environment sensing ability, several spore-based technologies have been developed (Table 15.1). Spores as a biosensor have been used for the detection of various microbial and nonmicrobial contaminants in different food stuffs. Because of the metal-binding properties of their surface protein, they have been exploited in the field of biosorption. Being resistant to harsh environmental conditions, spores have been used to develop probiotics that can easily pass through the acidic conditions of the stomach in order to reach the intestine. Spores have also been used for agricultural applications, in development of biocontrol and biofertilizers. There other useful applications including development of biopolymers, enhancing durability of building materials and biocatalysis, etc. Thus the unique inherent characteristics of spores have proved the spores as a valuable tool for fulfilling the need of current time in the area of food, environment and medicine. The presented information highlights about such breakthrough spore-based technologies. Moreover, the chapter also unfolds unlimited scope and hidden potential spores that could lead to many more demanding applications as per the need of times to come.

15.2 Microbial Spores

Microbial spores are ubiquitous in nature. Due to their inherent stability and resistance properties, they are distributed in different kinds of environments. They are found in soils; aquatic environment; extreme environments such as deserts,

Table 15.1 Applications of microbial spores

Field	Spores	Principle	Application	References
Biosorption	*Bacillus subtilis*	Genetically engineered surface proteins (Cot B) for enhanced metal-binding affinity	Bioremediation of heavy metals contaminated sites	Hinc et al. (2010)
Biosensors	*Bacillus megaterium*	Spore germination and enzyme inhibition	Pesticide detection in milk	Kumar et al. (2015)
	Bacillus stearothermophilus	Spore germination and DPA release	Antibiotic detection in milk	Kumar et al. (2006)
	Bacillus megaterium	Enzyme release and spore germination	*Listeria monocytogenes* detection in milk	Balhara et al. (2013)
	Cladosporium cladosporioides	Spore inhibition	Fungicides detection	Kanatiwela and Adikaram (2009)
Biocontrol	*Bacillus thuringiensis*	Endotoxins	Lepidopteran larvae	Whalon and McGaughey (1998)
	Pythium oligandrum	Applied as seed treatment	Damping-off disease caused by *P. ultimum* in sugar beet	Lewis et al. (1989) and Khetan (2001)
	Beauveria bassiana	–	*Anopheles gambiae*, mosquito control	Farenhorst et al. (2011)
Biopolymer	*Saccharomyces cerevisiae*	Presence of chitosan in spore coat layer	Bioadsorption and enzyme immobilization	Zhang et al. (2014)
Biofertilizer	*Frankia*	Nitrogen fixation	*Casuarina cunninghamiana*	Lalonde and Calvert (1979) and Burleigh and Torrey (1990)
Medicine	*Bacillus subtilis* LTB antigen (spore-based delivery system)	Chromosomal-encoded C-terminal fusion with spore coat CotC protein	*Escherichia coli* (ETEC); induction of serum and faecal antibody responses	Mauriello et al. (2004)
Microalgal industry	Fungal strains, isolated from compost, straws and soil	Algal flocculation	Waste water treatment and biofuel production	Muradov et al. (2015)

hydrothermal sites and arctic ices; food system; etc. Spores from different microbial groups are also known to form associations with insects, animals, plants and other organisms. Most of the times, such associations also help them to carry out their entire life cycle comprising of germination-growth-sporulation, within the animal host (Cutting and Ricca 2014).

Being a survival strategy, spores are well resistant to varying ranges of temperature and pressure, UV radiation and many noxious chemical substances. A number of theories were proposed in earlier times during 1970–1975, to understand the resistance of microbial spores. These theories included “contractile cortex theory”, the “expanding osmoregulatory cortex theory”, the “anisotropic swollen cortex theory”, the “high-polymer matrix theory” and the “calcium-dipicolinate complex theory”. These theories were established based on the heat resistance mechanism as determined by observations from analysis of spore components and electron microscopy (Murata 1993). Recent advancements in biochemical, genetic and molecular techniques have further contributed to the better understanding of structural and functional properties of spores from different microbial groups.

The process of spore formation is known as sporulation. It involves progression through different stages including commitment to sporulation, chromosome segregation, sporulation-specific cell division, differential gene expression and specific signal transduction mechanisms (Cutting and Ricca 2014). Spores can remain in dormant form for long time periods; however they persistently scrutinize their environment for favourable conditions, the presence of germinants that trigger germination. Furthermore, spores possess the unique properties to recognize stereoisomerically distinct forms of germinants (Tehri et al. 2017). During germination initiation of metabolism takes place resulting in ATP formation and synthesis of RNA and proteins. Eventually replication of DNA results in vegetative cell (Setlow 1983; Paidhungat and Setlow 2002). A new vegetative cell is able to grow, to duplicate and eventually to sporulate again. This exclusive biphasic phenomenon of spores holds great potential to form the basis of development of several spore-based techniques, mentioned below under section of industrial applications.

15.3 Industrial Applications of Microbial Spores

Spores of various types of microorganisms, especially bacterial spores, have been evaluated for their potential for industrial applications in several fields (Fig. 15.1). A number of such spore-based technologies developed till date have been discussed below.

15.3.1 Biosensors

Spores from microorganisms such as bacteria and fungi have been used to develop sensing systems that can easily detect the presence of various microbial and nonmicrobial contaminants in food, clinical and environmental samples. Spore-based

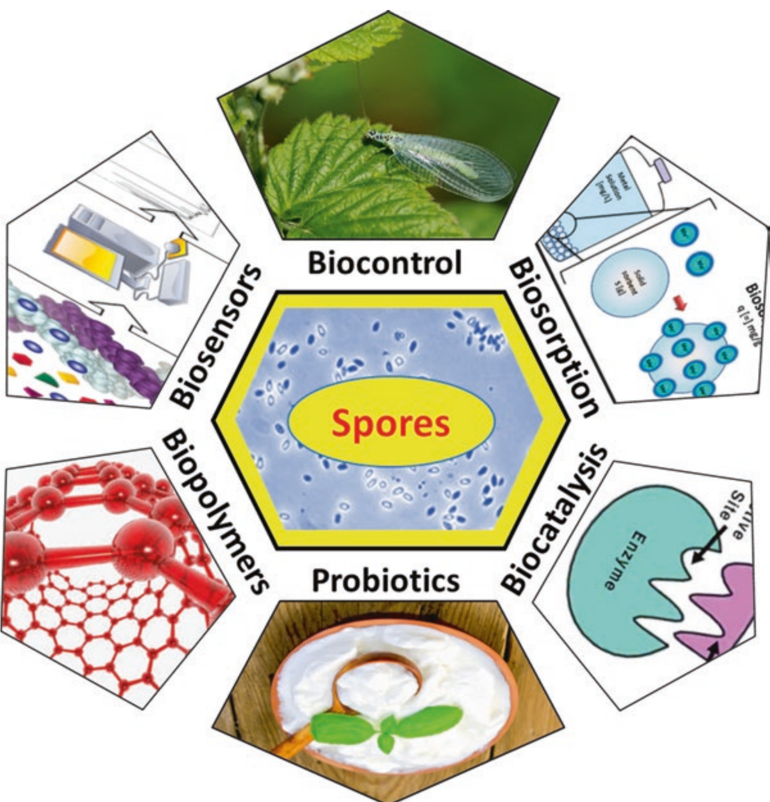

Fig. 15.1 Applications of microbial spores

sensors are cost-effective, have long shelf-life and give a real-time response. Keeping in view, several spore-based biosensors have been developed to target different analytes. Spore-based sensors have been developed to detect β-lactam and several other groups of antibiotics in milk (Das et al. 2011; Kumar et al. 2014). The developed assays are chromogenic and work on the principle of inhibition of spore germination in the presence of antibiotic residues. Furthermore, spores have also been used to target the presence of pathogens that are of public health significance. One such sensor has been developed for detection of *Listeria monocytogenes* in milk. This is a two-stage assay: the first stage is pre-enrichment and the second stage includes the detection of pathogen with the help of *B. megaterium* spores. The assay works on the principle of conversion of complex sugar into simpler form with the help of a marker enzyme present in *L. monocytogenes.* The resulting simpler sugar acts as a nutrient germinant for spores of *B. megaterium.* The germination in spores, indicating the presence of pathogen, is assayed by means of a fluorogenic enzymatic assay (Balhara et al. 2013). In addition to bacterial spores, spores of fungi have also been used to develop spore-based sensing systems. A convenient, cost-effective and sensitive fungal spore-based thin layer chromatography bioassay

has been developed for detection of fungicidal residues. The assay has successfully been used for detection of fungicides on tomato. The assay works on the principle of *Cladosporium* spore inhibition in the presence of fungicides. Thus the presence of fungicides is indicated by the lack of aerial mycelium (Kanatiwela and Adikaram 2009).

15.3.2 Biopesticides

The biopesticides can be defined as the types of pesticides which have biological origin. They are usually derived from animals, plants, bacteria and certain minerals. The most common type of biopesticides is based on microorganisms that are pathogenic to the pest to be killed. They can be classified as biopesticides, biofungicides and bioherbicides. The use of biopesticides offers several advantages over chemical pesticides. Biopesticides are safe to humans and the environment, are cheap and have more target specificity. Spores from bacteria and fungi are promising candidates for their use as biopesticides. Spores of *Bacillus thuringiensis* (BT) are widely used in agriculture to target insects and pests. BT is known to produce crystal protein inclusions, with toxic insecticidal properties, during the process of sporulation. Apart from this, various subspecies of BT have been identified with varying levels of toxicity against different insects; e.g. the subspecies aizawai is effective against moths, kurstaki is for moths, israelensis is for mosquitoes and flies and tenebrionis is for beetles (Arora et al. 2016). The commercial products based on BT are available in powdered form containing chiefly dried spores and toxin crystals. Spores of *B. sphaericus* are also known to develop a parasporal body during the process of sporulation. It has toxic properties against mosquito's larvae.

15.3.3 Biosorption

The use of biological agents to bind and accumulate pollutants such as heavy metals is known as biosorption (Fourest and Roux 1992). It offers a low-cost, high efficiency, cost-effective treatment for contaminated sites and regeneration of adsorbed materials like recovery of metals (Kratochvil and Volesky 1998a, b). In this context, bacterial spores have also been evaluated to act as biosorbents. Spores of a marine *Bacillus* sp. strain (SG- 1) have been found to bind and oxidize Mn (II) and Co (II) (Lee and Tebo 1994). The Cot B surface protein in spore coat of *B. subtilis* has been engineered at molecular level for expression of 18 residues of histidine fused to promoter and its N-terminal part. The resulting recombinant spores were reported to exhibit greater binding affinity of spores to Ni ions than that of wild type (Hinc et al. 2010). Thus the metal-binding properties reveal the unique nature of bacterial spores to carry out bioremediation in heavy metal-contaminated sites. Furthermore, the peculiar properties such as resistance and stability against harsh environmental conditions make the spores potentially more interesting as a bioremediation tool.

15.3.4 Biopolymers

Chitosan is a polymer made up of β-1, 4-linked D-glucosamine. It has unique adsorptive properties. It acts as a chelator for transition metal ions and has positively charged amino groups that can bind molecules bearing negative charge. Because of these characteristics, chitosan finds several industrial applications in food, chemicals, environment and medicine. Production of chitosan by chemical means faces many challenges including generation of hazardous waste. In order to solve this issue, various biological approaches have been studied for the production of chitosan (Shahidi et al. 1999; Lee et al. 2009; Kardas et al. 2012). Spores of budding yeast *Saccharomyces cerevisiae* have successfully been used for production of chitosan beads. The spore wall of yeast has outer layer of dityrosine and second layer of chitosan. The gene coding for dityrosine, i.e. DIT1 gene, was removed leaving chitosan layer at the spore surface. Thus resulting spores resemble chitosan beads. The practical utility of such spore-based chitosan beads for removal of heavy metals was also examined.

Spores with dityrosine layer were found capable of adsorbing heavy metals such as Cu(II), Cr(III) and Cd(II). However increased rate of absorption was reported upon removal of dityrosine layer. Mutants with removed chitosan layer demonstrated reduced adsorption properties. This suggests the potential of chitosan from spores to act as an adsorbent. Chitosan can easily undergo chemical modifications, and therefore spores acting as chitosan beads can also be used as a carrier for enzyme immobilization (Zhang et al. 2014). Thus wild-type and mutant yeast spores both can be used as chitosan beads that can further find a number of applications in different fields.

15.3.5 Biocatalysis

Spores are considered as dormant and metabolically inactive, but at the same time they have been reported to contain all enzymes similar to those found in vegetative cells (Shigematsu et al. 1993). This suggests that microbial spores are unique bags of enzymes and have the potential to act as biocatalysts. In reference to these conclusions, endospores of *B. subtilis* and ascospores of *S. cerevisiae* have been evaluated for the biocatalytic activities. Two enzymes, namely, adenosine 5′-triphosphatase and alkaline phosphatase, from the spores of bacteria and yeast, respectively, have been used as model enzymes to assess their activity for industrial application. Spores were found to express both enzymes at a significant rate when subjected to physical (sonication or electric field pulse) and chemical (organic solvents or detergents) treatments. Furthermore the spore-based catalysis was used to produce chemicals in bioreactor systems. The use of spores as a source of enzymes was found to be advantageous over vegetative cells. This is because when vegetative cells are lysed to release enzymes for accomplishment of biocatalysis, enzymes come in contact with several biophysical and biochemical factors making them inactive. On the other hand, when spores are used, enzymes can remain stable in

spores in bioreactor system. Moreover, spores have also been successfully immobilized for continuous utilization to produce useful chemicals (Murata 1993). Hence, this finding indicates the superiority of spore (bacterial and fungal groups) usage over vegetative cells for their application to bioreactor systems in order to work as a biocatalyst.

15.3.6 Building Material

One of the most commonly used building materials is concrete. However cracks in concrete are a major problem. Cracks in concrete generally occur due to freeze thawing, shrinkage and mechanical forces. Ingression rate of corrosive chemicals like water and chloride ions in the structure of concrete gets increased (Meldrum 2003). In order to improve the strength of cement concrete, an attempt based on the use of bacterial spores has been made. Dormant spores of *B. sphaericus* have been incorporated in concrete matrix. Entry of water in the concrete begins the process of activation of bacterial spores. This results in the precipitation of calcium carbonate by the process of germinated spore-mediated metabolic activities (Gavimath et al. 2012). Thus this work clearly represents the potential application of microbial spores in improving the strength and durability of concrete, a building material.

15.3.7 Probiotics

The term "probiotics" means "for life". It has been derived from a Greek word (Reid et al. 2003). These are defined as, "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2005). Bacterial spores mainly those belonging to genus *Bacillus* have widely been used as probiotics for humans, animals and feed supplements. Probiotics aid to an improved digestion in host (le Duc et al. 2004; Hong et al. 2005). Administration of probiotics in the form of spores is advantageous. As being resistant to acidic conditions, spores upon ingestion can easily pass through the acidic conditions of stomach. They reach the intestine, and the conditions prevailing over therein are less acidic pH, nutrient support germination and subsequent cell growth (Cutting 2011). Several clinical trials on the human and animal models have been done to study the effect of spore-based probiotics. As a result oral administration of *Bacillus* spores was found to stimulate the immune system by triggering both specific humoral and cell-mediated immune responses (Suva et al. 2016). Furthermore, a study carried out with rabbits administered orally with spores of *B. subtilis* has shown the significant contribution of spores in development of the gut-associated lymphoid tissue (GALT) than other commensal bacteria (Rhee et al. 2004).

The spores of *Bacillus subtilis* have inherent properties of acting as probiotics, but addition of certain proteins, such as feed enzymes, on the surface of spores can further improve them for feed applications. Feed enzymes are generally added to

feed in order to enhance the digestion of animals. But to retain their activity while passing through stomach acids to reach the gut is very challenging. To solve this issue, inner coat proteins OxdD of spores have been engineered genetically to display the feed enzyme phytase on the surface of *B. subtilis* spores (Potot et al. 2010). Phytase is an enzyme that acts on the phytate-bound phosphorus, releasing free phosphorus and thus enhancing the nutritional value of feed.

Thus the spore-based probiotics offer several advantages such as prolonged persistence in the GI tract, the formation of robust biofilms, rapid sporulation and the stimulation of innate immune responses, room temperature stability without the need of refrigeration, etc. (Chen et al. 2014). These distinctive properties have led to their faster commercialization.

15.4 Current Status and Future Perspectives

The presented information uncovers the vast potential of microbial spores for their industrial applications. Spore-based technologies offer several advantages: (1) amenable to incorporation into portable devices, thus facilitating their use in field applications; (2) versatile performance during multiple germination and sporulation cycles, which further enhances their potential for reusability; (3) cost-effectiveness; (4) a wide range of aforementioned applications ranging from food to medicine; and (5) inherent stability of resisting harsh unfavourable environmental conditions. However, to get advantages of such spore-based applications, preference is usually given to those microbial spores (a) with the availability of detailed genetic and structural information, (b) that can be easily undergo genetic manipulation, (c) that have the safety record for their use on humans and animals (probiotic, medicine) and food systems and (d) that have potential robustness and heat stability. Till date, many such spore-based technologies mainly in the area of biosensing, biocontrol, biofertilizers and probiotics are available commercially, and others are in the queue of technology transfer process.

The use of spores to a wide range of applications in various fields has a bright future ahead. The research work to explore the unseen potential of microbial spores is still in continuation process. One such research work is going on at Harvard University which is attempting on the use of *B. subtilis* spores for generating electricity. Recently the forensic and archaeological value of fungal spores has also been recognized (Hawksworth and Wiltshire 2011), and related work is in progress at the University of Southampton. Apart from this, spores of bacteria have also been explored to provide possible mechanism for moving life among worlds and creating a new research area (Dehel 2006).

Thus unique features of spores make them an attractive tool for broad range of utility in various fields of science and technology. Furthermore, modern molecular-based studies are providing new paradigm on characteristics (biochemical and biophysical) of microbial spores which may further aid to more promising new applications.

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16 Insight into Compatible Solutes from Halophiles: Exploring Significant Applications in Biotechnology

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Abstract

Halophiles accumulate a restricted range of highly soluble low molecular weight molecules termed compatible solutes, which helps them to cope up with environments of elevated osmolarity. In addition to their stabilizing effects, compatible solute contributes significantly in different biotechnological applications. These include stabilization of biomolecules, stress-protective and therapeutic agents, in cosmeceuticals and pharmaceuticals, as a cryoprotectant of microorganisms, and increasing osmotolerance in non-halotolerant organisms by transforming genes for their synthesis. High solubility and low molecular weight of osmolytes make them potential candidates for bioprocessing and as an attractive proposition toward commercialization. We summarize here the current state of knowledge and applications of compatible solutes in biotechnology.

Keywords

Compatible solutes · Cryoprotectant · Halophiles · Osmolarity · Bioprocessing · Stress-protectant

16.1 Introduction

Microorganisms have evolved a remarkable range of mechanisms to adapt to environmental changes, such as increasing osmolarity. To cope with environments of elevated osmolarity or low water activity (a_w), halophiles adapt two mechanisms: "the salt-in-cytoplasm strategy" where they accumulate molar concentration of KCl

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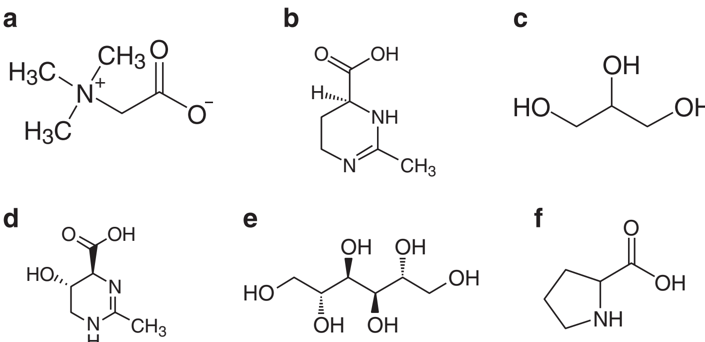

Fig. 16.1 Examples of some compatible solutes. (**a**) Glycine betaine (usually abbreviated as betaine), (**b**) ectoine, (**c**) glycerol, (**d**) hydroxyectoine, (**e**) mannitol, (**f**) proline. Ectoine and hydroxyectoine also known as THPs (tetrahydropyrimidines) have structural similarity with pyrimidine bases

in concentration higher than extracellular NaCl to maintain a turgor pressure and "the organic osmolyte strategy" where they accumulate nonionic, highly water-soluble organic osmolytes. These osmolytes are responsible for osmotic balance and at the same time compatible with cell metabolism (Roberts 2005; Kurz 2008). Compatible solutes also known as osmolytes are a restricted range of low molecular weight molecules, which includes sugars, polyols, amino acids, and their derivatives such as betaines, ectoines, N-acetylated diamino acids, and N-derivatized carboxamides of glutamine (Fig. 16.1).

Properties of compatible solutes like high solubility, broad variation in concentration, interaction without affecting cellular metabolism, and being compatible at low molar concentration make them suitable candidates for bioprospecting (Malin et al. 1999; Brigotti et al. 2003). A list of their application starts with the stabilization of protein and cells (Louis et al. 1994; Hoeckstra et al. 1997; Barth et al. 2000), their relevance for bioremediation (Patzelt 2005), their application in cancer research or dermatology (Lindemose et al. 2005; Cornacchione et al. 2007; Heinrich et al. 2007), their being a natural component of food (Klein et al. 2007), their interface with nucleic acid (Mandal et al. 2004), and many more. In the present book chapter, we have tried to cover current state of knowledge and recent advancements that have been made in this field.

16.2 Osmolytes from Halophiles

Osmolytes fall into three categories: (1) zwitterionic solutes, (2) noncharged solutes, and (3) anionic solutes. These compatible solutes and their derivatives are highly water-soluble and low molecular weight osmoregulatory compounds. Their occurrence in halophilic and halotolerant bacteria is presented in Table 16.1.

Table 16.1 Examples of different categories of compatible solutes and their occurrence in moderate to extreme halophiles

Zwitterionic solutes	Occurrence	Noncharged solutes	Occurrence	Anionic solutes	Occurrence
Betaine	*Thioalkalivibrio versutus*, *Halorhodospira halochloris*, *Methanohalophilus portulcalensis*, *Methanosarcina thermophila*	α-Glucosylglycerol	*Synechocystis* sp., *Rhodovulum sulfidophilum*, *Pseudomonas mendocina*, *Stenotrophomonas*	β-Glutamate	*Halomonas elongata*, *Methanohalophilus portucalensis*, *H. salinarum*
Ectoine	*Halomonas elongata*, *H. variabilis*, *Vibrio cholerae*, and *V. costociola*	α-Mannosylglyceramide	*Rhodothermus marinus*	Hydroxybutyrate	*Photobacterium profundum*
Hydroxyectoine	*Halomonas elongata*, *Nocardiopsis halophila*	Sucrose	*Synechocystis* sp., *Anabaena* spp., *Proteobacteria*	Polyhydroxybutyrate	*Photobacterium profundum*, *Methylarcula marina*, and *M. terricola*

(continued)

Table 16.1 (continued)

Zwitterionic solutes	Occurrence	Noncharged solutes	Occurrence	Anionic solutes	Occurrence
Nε-Acetyl-β-lysine	*Methanosarcina thermophila*, *Methanothermococcus thermolithotrophicus*, *Methanohalophilus portucalensis*, *Methanohalophilus* sp.	Trehalose	*Actinopolyspora halophila*, *Desulfovibrio halophilus*, *Rhodothermus obamensis*, *Natrialba magadii*	Glucosylglycerol	*Agmenellum quadruplicatum*, *Methanohalophilus portucalensis*
β-Glutamine	*Methanohalophilus portucalensis*	N-Acetylglutaminylglutamine amide	*Ectothiorhodospira mobilis*	Mannosylglycerate	*Methanothermus fervidus*, *Rhodothermus marinus*

16.2.1 Zwitterionic Solutes

Zwitterionic solutes are neutral in nature, which are derivatives of amino acids and are formed as an intermediate product in cellular metabolism. These solutes are dipolar (with spatially separated positive and negative charges) and are excluded from the hydration shell of the macromolecules.

16.2.1.1 Betaine

Betaine is ubiquitous osmolyte, found diversely in all three kingdoms of life. Glycine betaine, also abbreviated as betaine, is a primary amine methylated to form quaternary amine (Imhoff and Rodriguez-Valera 1984). High solubility of this compound makes it an excellent compatible solute and is found diversely in different phylogenetic relationships. In most of the cells, betaine is transported into the cell from external medium via specific membrane proteins belonging to ABC superfamily of transporters. Very few halophiles (e.g., *Halomonas elongata*, *Actinopolyspora halophila*) are able to synthesize betaine de novo by oxidation of choline or methylation of glycine (Robertson et al. 1990; Nyyssölä et al. 2000).

16.2.1.2 Ectoine and Hydroxyectoine

Ectoine is one of the most broadly occurring osmolytes in halophiles and is most abundant osmolyte in nature. Ectoine is a cyclic tetrahydropyrimidine (1,4,5,6-tetra hydro-2-methyl-4-pyrimidinecarboxylic acid). It was first detected in the halophilic, phototrophic *Halorhodospira halochloris* (Galinski et al. 1985). Concentration of ectoine in the cell depends on the osmolarity of the medium and helps the cell to survive in high osmotic concentration. The ability to accumulate ectoine gives ecological advantage to an organism. The *Vibrio cholera* cells adapted to high osmolarity and outcompete nonadapted cells in both fresh and marine environment by accumulating ectoine and betaine (Pflughoeft et al. 2003).

A number of halophiles also synthesize an ectoine derivative 5-hydroxyectoine. Like ectoine, 5-hydroxyectoine serves as a compatible solute in vivo and is synthesized through direct hydroxylation of ectoine via the evolutionarily conserved enzyme ectoine hydroxylase (Bursy et al. 2007; Höppner et al. 2014). Hydroxyectoine exhibits stress-protective properties that are partially different from and sometimes exceed those of ectoine. It counteracts the deleterious effects of high osmolarity on cell physiology and loss of cell water (Bursy et al. 2007; Tanne et al. 2014).

16.2.1.3 Nε-Acetyl-β-Lysine and β-Glutamine

Extremely halophilic archaebacteria survive in extreme saline conditions by accumulating potassium, such as *Halobacterium* and *Halobium* species (Javor 1989). Halophilic methanogenic archaebacteria, however, accumulate β-amino acids as compatible solutes in response to external NaCl concentration. β-Amino acid derivatives Nε-acetyl-β-lysine and β-glutamine were found to accumulate in *Methanosarcina thermophila* and *M. cariaci* and species of *Methanohalophilus* (Sowers et al. 1990; Martin et al. 2001). These two neutral amino acid derivatives appear to be a unique characteristic of methanogenic archaebacteria.

16.2.2 Noncharged Solutes

16.2.2.1 α-Glucosylglycerol and α-Mannosylglyceramide

Some solutes are polar in nature but lack formal charges. These solutes are well characterized in halophilic organisms as well as in eukaryotes. An example of this is glycerol, which is found prevalent in *Dunaliella* (Borowitzka and Brown 1974). Accumulation of glycerol as an osmolyte is also reported in halotolerant yeast *Debaryomyces hansenii* as well as in black yeast *Hortaea werneckii*. It helps to adapt these eukaryotic organisms to high level of NaCl concentration that reduce cell turgor and induce osmotic stress (Petrovic et al. 2002).

Soluble carbohydrates such as glucose, sucrose, fructose, and fructans significantly contribute to mechanism of adaptation to salt stress. The sugars with more reducing ends are reactive, and these noncharged solutes probably react with surface amino acids of proteins. However, this is avoided by making glycosidic bonds with small neutral molecules, such as glycerol or glyceramide (Silva et al. 1999). *Proteobacteria*, *Stenotrophomonas* and *Rhodothermus marinus*, accumulate α-glucosylglycerol and α-mannosylglyceramide, respectively, to cope with increased salinity (Silva et al. 1999; Roder et al. 2005). These groups of bacteria are known for their large biotechnological potential, one of which is production of glucosylglycerol.

16.2.2.2 Trehalose and Sucrose

Nonreducing sugars, such as trehalose, are also used as an osmolyte by many groups of bacteria. In *Actinopolyspora halophila*, in *Chromohalobacter israelensis*, and in the sulfate-reducing bacterium *Desulfovibrio halophilus*, trehalose is an important solute when cells are grown in increased osmotic concentration (Regev et al. 1990; Nyyssölä and Leisola 2001). Disaccharides, principally sucrose, are used by some halotolerant and halophilic bacteria to enhance growth in higher NaCl. The use of sucrose as an osmolyte is usually associated with low-salt-tolerant strains. The *Synechocystis* sp. strain PCC 6803 tolerates up to 1.2 M NaCl by transporting sucrose from external medium. Sucrose is more commonly used for survival in stationary phase. These observations preferably suggest the use of sucrose in metabolic pathways that are active when cell face starvation conditions in stationary phase (Deplats et al. 2005).

16.2.2.3 N-Acetylglutaminylglutamine Amide (NAGGN)

N-Acetylglutaminylglutamine amide (NAGGN) is also known as class of osmotically regulated peptides. NAGGN is a prominent example where a peptide is used as an osmolyte; most of the peptides synthesized by bacteria are only antibiotics. NAGGN is first reported as dipeptide in stressed *Rhizobium meliloti*, where it is found to overcome osmotic stress (Smith and Smith 1989). Accumulation of NAGGN is proportional to the concentration of NaCl and chemical composition of growth medium (D'Souza-Ault et al. 1993).

16.2.3 Anionic Solutes

Anionic solutes are sort of counterions that in many cases appear to be modified forms of organic osmolytes found in less-salt-tolerant species. In many cases, uncharged organic osmolytes gain negative charge through acquisition of carboxylate, sulfate, or phosphate group. An example is commonly occurring osmolyte glycerol that appears as diacylglycerol (DAG), which has net charge of minus one.

16.2.3.1 β-Glutamate

β-Glutamate was first to be observed in methanogenic archaebacteria in response to osmotic stress. β-Glutamate is synthesized de novo, and it exhibits slower turnover rate as compared to other osmolytes (Robertson et al. 1992a, b). While most studies found β-glutamate in methanogenic bacteria, it has also been detected in the gram-positive bacteria *Nocardiopsis halophila* (DasSarma and Arora 2002).

16.2.3.2 Hydroxybutyrate and Polyhydroxybutyrate

Hydroxybutyrate and polyhydroxybutyrate are normally used as a carbon source reservoir in a number of bacteria, including *Methylarcula marina* and *Methylarcula terricola* (Roberts et al. 1990; Doronina et al. 2000) and in the deep-sea bacteria *Photobacterium profundum* SS9 (Martin et al. 2002). Polyhydroxybutyrates in the deep-sea bacteria play an interesting role in combating high atmospheric pressure and are accumulated as major solutes. Since these osmolytes increase with osmotic and hydrostatic pressure, they are termed as piezolytes (Martin et al. 2002).

16.2.3.3 α-Glucosylglycerate and α-Mannosylglycerate

As discussed earlier, intracellular solutes with negatively charged carbohydrates are rarely found. Two such solutes α-glucosylglycerate and α-mannosylglycerate are detected in species of many bacteria including *Rhodothermus*. These solutes engage their reactive end group of sugars by forming glycosidic bonds with hydroxyl group of glyceric acid. Their accumulation in the cell depends on the type of stress conditions. Under increased temperature, anion mannosylglycerate was found to predominate and the neutral α-mannosylglyceramide found to accumulate under increased NaCl concentration (Silva et al. 1999).

16.3 Molecular Interaction and Stabilization by Osmolytes: Theories

Biotechnological potential of compatible solutes lies in their ability to interact and stabilize biomolecules in stress conditions. Applicability of compatible solutes for bioprocessing and commercialization pertains to two theories: (1) direct solute-macromolecule interactions and (2) macromolecular stability mediated by solute-induced changes in water structure.

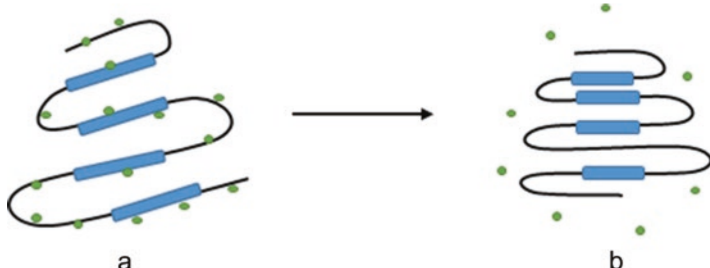

Fig. 16.2 A newly formed protein (native protein) when exposed to high osmotic concentration would denature. Interaction of compatible solute (shown in light green circle) and exclusion from protein surface leave protein hydrated. Induced hydration by osmolytes makes protein fold compactly with less exposed area than denatured protein. (**a**) Interaction of osmolytes with native protein, (**b**) exclusion of osmolytes and compact folded protein

16.3.1 Compatible Solute Interaction with Proteins: Exclusion and Hydration

At high osmotic concentration, osmolytes in the cell consistently compete with water molecules for interaction with protein surfaces. This energetically unfavorable interaction promotes exclusion of osmolyte from the surface of the protein, eventually making protein hydrated (Fig. 16.2). This increased osmotic pressure induced by osmolytes preferentially triggers compact folded protein, with less exposed area than denatured protein (Liu and Bolen 1995; Plaza di Pino and Sanchez-Ruiz 1995; Timasheff 2002). Thermodynamically, osmolytes raise the free energy level of the native protein in the unfolded state. This increased energy level makes the protein to fold compactly (Fig. 16.3). Polypeptide chains in proteins interact differentially with water and osmolytes. Compared to water, osmolytes have more unfavorable interaction with polypeptide backbone (Bolen and Baskakov 2001). Native proteins have more exposed polypeptide backbone, which allow more solute to interact, shifting the energy equilibrium that promotes protein folding (Cioni et al. 2005).

16.3.2 Osmolyte-Induced Changes in Water Structure

The ability of different ions to bind water depends on their interaction with water molecules. The Hofmeister series or lyotropic series is a classification of ions in order of their ability to salt-in or salt-out proteins. Ions in the Hofmeister series reflect the ability of different ions to bind water (Baldwin 1996; Kunz et al. 2004). Ions which exhibit strong interaction to water molecules are called “kosmotropes” (order makers), while those which exhibit weaker interaction with water are called “chaotropes” (disorder makers). Kosmotropes have a tendency to bind strongly with

Fig. 16.3 Osmolytes stabilize native proteins from denaturation by thermodynamically raising energy level of the unfolded state. Figure showing the difference in energy level of folded and unfolded protein in the presence of osmolytes and in its absence. Where U, unfolded; F, folded; aq, aqueous solution; ΔG, free energy; S, solution containing osmolytes

water than protein and hence preserve preferential hydration of protein. These solutes prefer interaction with water than protein surface. On the other hand, chaotropes preferably displace water from the protein surface leading to the destabilization of protein.

Many osmolytes behave prominently to the ions of Hofmeister series, such as amino acid resembling ammonium acetate and the methylamines being functionally similar to quaternary ammonium ions (Yancey et al. 1982). Collins and co-workers set out that ions of Hofmeister series are arranged according to the hydration number of the ion: the more hydrated an ion, the greater the stabilization of macromolecules (Collins and Washabaugh 1985). Osmolytes, which are more hydrated with dense layer of water around it, are not able to penetrate solvation layer around other solutes (macromolecules), eventually destabilizing macromolecule.

16.4 Biotechnological Applications of Compatible Solutes

Osmolytes from halophiles have been employed for biotechnological applications by industries for a wide range of uses. This is possible because their properties can be exploited in vitro or in vivo. Use in therapeutics, as a cryoprotectant, in cosmeceuticals and pharmaceuticals, enhancing PCR, stabilization of proteins and nucleic acids, and generation of stress resistance in non-halotolerant organisms are some of the prominent applications of osmolytes. Table 16.2 summarizes the biotechnological applications of osmolytes in different areas.

Table 16.2 Summary of biotechnological applications of some osmolytes

Osmolyte	Biotechnological applications	References
Betaine	Used as a cryoprotectant in the preservation of microorganisms during long-term storage	Roberts (2005)
	In cosmeceuticals as osmoprotectant to prevent skin protein from denaturation	Desmarais et al. (1997)
	Used for the treatment and prophylaxis of adipose infiltration of the liver	Detkova and Boltyanskaya (2007)
	Has anticoagulant property, prevents thrombus formation, and decreases the probability of heart attacks, infarctions, and strokes	Messadek (2005)
	Useful for many qualitative and quantitative multiplex PCR	Weissensteiner and Lanchbury (1996)
	Generation of stress resistance in non-halotolerant organisms	Alia Hayashi et al. (1998), Holmstrom et al. (2000), and Rontein et al. (2002)
Ectoine	In cosmeceuticals as an osmoprotectant that saves skin from UV radiations	Desmarais et al. (1997)
	Enhances PCR amplification of GC-rich template	Schnoor et al. (2004)
Glycerol and sucrose	Protects normal cells from toxic effect of chemicals and plasma during cancer treatment	
Trehalose	Prevents denaturation by providing thermostability to proteins and enzymes	Santosh and Costa (2001) and Borges et al. (2002)
	Prevents amyloid formation of insulin in vitro	
Proline	Stabilization of nucleic acids	Chadalavada et al. (1997), Draper et al. (2005), Lambert and Draper (2007), and Schwinefus et al. (2007)

16.4.1 Cryoprotectant of Microorganisms

Maintaining viability of microorganisms during freezing is a major task when microorganisms are stored for long durations. This can be overcome by using cryoprotectant. Among different cryoprotectants, the ability of betaine has been studied widely for diverse group of bacteria (Roberts 2005). When compared to other cryoprotectants such as trehalose and serum albumin, betaine is found to be better, particularly under long-term storage. Some studies have reported betaine as effective osmolyte for liquid nitrogen storage of halophilic archaebacteria and neutrophilic Fe-oxidizing bacteria (Cleland et al. 2004). During cryopreservation, some microorganisms such as *Listeria monocytogenes* transport betaine by Na^{+}-linked system (Ko et al. 1994; Patchett et al. 1994; Gerhardt et al. 1996) or by an ATP-driven system (Ko et al. 1994) into the cytoplasm. This stimulates the growth rate and helps in long-term survival.

16.4.2 Applications in Cosmeceuticals and Pharmaceuticals

Specific property of osmolytes to protect the cell in stress conditions has been used in the cosmeceutical industries. Ectoine has been shown to protect skin from UV-A-induced cell damage (Desmarais et al. 1997). Du Point, a US-based cosmeceutical industry, is using betaine and inositol as an osmoprotectant to protect cells from dehydration and to prevent skin proteins from denaturation. Cosmeceutical industries like RonaCare™, Merck KGaA, and Darmstadt are using ectoine as a moisturizer in cosmetics and skin care products.

Unique properties of osmolytes such as protective metabolic, antioxidant, and protecting macromolecules are now being explored in health care. Osmolytes such as glycerol and sucrose that belong to polyhydric alcohols are used to protect normal cells from toxic effect of chemicals and plasma during cancer treatment. During cancer therapies, the use of osmolytes has shown to protect tissues against vascular leak syndrome, a severe side effect of anticancer agents, an example where osmolytes are used as a tissue protectant.

Some osmolytes have excellent therapeutic potential; betaine is used in the initial stage of cirrhosis for the treatment and prophylaxis of adipose infiltration of the liver (Detkova and Boltyanskaya 2007). Apart from this, it decreases the side effects of anti-inflammatory preparation, has anticoagulant property, prevents thrombus formation, and decreases the probability of heart attacks, infarctions, and strokes (Messadek 2005).

16.4.3 Chemical Chaperones for Protein Folding

Environmental stresses trigger alteration in protein folding. Stress conditions like high temperature and pH lead to the denaturation of the proteins resulting in the unfolding of proteins (Fändrich et al. 2001). A number of structurally different proteins misfold during high stress conditions. This is particularly reported for amyloidogenic proteins, leading to diseases such as Alzheimer’s and Parkinson’s disease, mad cow disease, and others (Harper and Lansbury 1997; Murphy 2002). Nucleation, elongation, and saturation of amyloidogenic proteins under stressful conditions lead to partial unfolding of proteins. Osmolytes such as trehalose and betaine contribute to thermostability of enzymes and have propensity to stabilize native confirmation of proteins (Santosh and Costa 2001; Borges et al. 2002). These properties of osmolytes have been reported to prevent amyloid formation of insulin in vitro.

Protein-stabilizing properties of osmolytes have also been explored for high yield of functional folded cytoplasmic proteins (Fig. 16.4). Cells grown under moderate saline conditions and supplemented with betaine can accumulate large amount of target proteins in periplasm (Barth et al. 2000). This finding provides direction for designing molecules for better treatment of protein structural deformity-related diseases and for high yield of functional proteins at industrial scale.

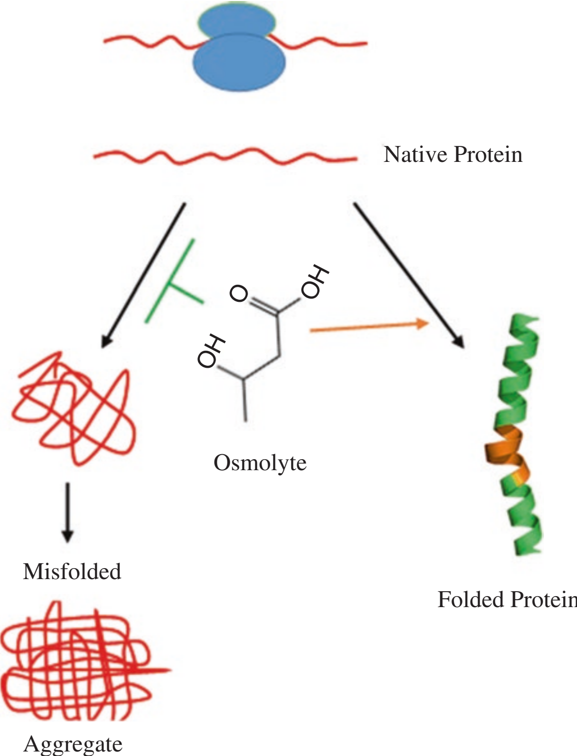

Fig. 16.4 Osmolytes act as chemical chaperones stabilizing the native protein. Protein when exposed to increased osmolarity is misfolded and eventually aggregates. Osmolytes stabilize native conformation of protein to yield functional folded cytoplasmic protein

16.4.4 Stabilization of Nucleic Acids

Properties of osmolytes to interact with intracellular molecules can modulate many cellular processes including regulation of protein, protein-protein interaction, and protein-DNA interaction (Singh et al. 2011). Osmolytes have different effects on RNA that relies on secondary or tertiary structures. RNAs have high negative charge density and hence strong interaction with ions (Draper et al. 2005). Osmolytes affect stability of RNA by modulating effective concentration of ions (Lambert and Draper 2007). Glycine betaine stabilizes RNA pseudoknot tertiary structure with no changes in structure up to 40 mm NaCl. It interacts more strongly with surface area exposed on unfolding of GC-rich bases (Schwinefus et al. 2007). Many osmolytes destabilize the DNA double helix and lower the T_m of DNA. For example, proline considerably decreases T_m of DNA and partially counteracts the effect of sodium chloride and spermidine on DNA stability. This helps in nullifying the deleterious effect of NaCl on DNA stability and hence protects double helix from denaturation (Rajendrakumar et al. 1997).

16.4.5 Enhancing PCR

DNA templates with high GC content require high T_m (melting temperature) and are subjected to constant association and dissociation during PCR. Additions of molecules that increase thermostability are shown to be useful in PCR amplification. Several osmolytes including ectoine and betaine are found to enhance PCR amplification of GC-rich DNA templates. Particularly, ectoine has outperformed other PCR enhancers by reducing DNA T_m significantly (Schnoor et al. 2004)

In diagnosis of heritable diseases, most PCR assay involves co-amplification of an internal control of a several alleles at a given locus. In such a multiplex reaction, amplifiers that are more sensitive outcompeted under suboptimal PCR conditions leading to false-negative results. Adding dsDNA-stabilizing additives such as betaine has shown to minimize false-negative result-inducing conditions. Betaine is found to be an efficient solute in PCR and useful for many qualitative and quantitative multiplex PCR (Weissensteiner and Lanchbury 1996).

16.4.6 Generation of Stress Resistance in Non-halotolerant Organisms

Development in the molecular biology has provided immense possibility to transform genes from one species to another. Insertion of genes for osmolytes to non-halotolerant organisms would increase their ability to tolerate stress. Plants are often exposed to water scarcity that would concentrate salts. Insertion of genes for in vivo synthesis of osmolytes in plant has significantly improved tolerance to stress conditions. As an example, salt tolerance in *Arabidopsis thaliana* is found to improve notably by transforming choline oxidase gene (which is needed to synthesize betaine) from *Arthrobacter globiformis* (Alia Hayashi et al. 1998). Transgenic lines of tobacco have also been constructed by transforming betA and betB genes from *E. coli*; this abatement exhibits better cold and salt resistance in tobacco plant (Holmstrom et al. 2000). However, accumulation of osmolytes in transgenic plants is limited to a certain extent and does not lead to high accumulation of osmolytes with increase in osmolarity. Further development is needed to overcome these limitations (Rontein et al. 2002).

16.5 Concluding Remarks

Upon exposure to abiotic stress, it is commonly observed that cell adapts by modulating cellular machinery. Halophiles combat these stresses by de novo synthesizing compatible solutes or accumulating it from external environment. While exploring biotechnological applications of osmolytes, it is important to understand the mechanism of osmolyte function and selective rationale for osmolyte pattern and types and their occurrence.

The use of osmolytes at industrial scale is expanding; after reorganizing their potential, they are widely used for different biotechnological applications including stabilization of protein and DNA, as a cryoprotectant of microorganism during long-term storage, in cosmeceuticals and pharmaceuticals, as PCR enhancers, and in generation of stress resistance in non-halotolerant plants. Though promising in ways, production and purification of osmolytes for industrial scale may be challenging. As these challenges are met, the use of osmolytes for bioprospecting will continuously expand.

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17 Riboswitches as Molecular Tools for Microbial Bioprospecting

Jeena Gupta and Tasaduq Peerzada

Abstract

The ability of the microbes to adapt and survive under different environmental conditions depends upon their ability to precisely regulate gene expression in response to external stimuli. In microbes, this generally accounts for regulation of transcription at DNA level via direct interaction of transcription factors, activators or repressors, etc. that can detect the presence/absence of certain metabolites. However, recent advancements have detected the presence of certain control elements on mRNA, which possess the ability to detect small metabolites and evoke a regulatory effect on protein expression. These cis-acting RNA regulatory domains present on untranslated mRNA are called riboswitches that have a characteristic structure and have the ability to directly bind small molecules primarily metabolites but can also sense temperature and small metal ions, binding of which leads to a conformational change. Regulation by riboswitches can either occur at the level of transcription by formation of terminator/anti-terminator structures or initiation of translation by formation or depletion of the ribosomal binding sites. Riboswitches are emerging as important contributor to control prokaryotic gene expression controlling a multitude of biologically important pathways, including bacterial vitamin and amino acid biosynthesis. This accurate and efficient mechanism can be employed to control the characteristic ligand-responsive gene expression and thus holds the capacity to be used as a bio-tool or biosensor paving a completely new way for generation of inducible expression systems. The ligand mimetics and microbial riboswitches serve as tools for bioprospecting since they are being used as antimicrobial agents. This chapter will cover the recent advances, current achievements, and limitations faced by the riboswitch technology to be used as a bio-tool.

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Gene expression · Transcription · Riboswitches · mRNA and bio-tool

17.1 Introduction

"Riboswitch" is a term used for special RNA sequences which control gene expression in response to temperature changes, binding of tRNA or metal ions without requiring protein factors (Johansson 2009; Klinkert and Narberhaus 2009; Gutiérrez-Preciado et al. 2009; Cromie et al. 2006; Dann et al. 2007). These are noncoding RNA domains in messenger RNA (mRNA) which undergoes allosteric structural changes after binding small metabolites and control transcription or translation (Mandal et al. 2004; Coppins et al. 2007; Roth and Breaker 2009).

The first RNA-based regulation then called as "riboregulation" was discovered by Charles Yanofsky in concern to regulate tryptophan biosynthesis at mRNA level (Yanofsky et al. 1996). Dr. Tina Henkin first observed a conserved sequence in the 5′ region of S-box gene family which regulates the biosynthesis of methionine and cysteine with potential to form secondary or tertiary structures predicted by computer models. However, the term "riboswitch" was first used in 2002 by Dr. Ronald Breaker, who observed the property of mRNAs to bind metabolites directly without involving proteins to regulate membrane transportation of coenzyme, B_{12}. Since then there is immense progress in riboswitch research particularly in the last decade with approximately three new classes of riboswitches being discovered per year (Roth and Breaker 2009).

Riboswitches form cis-acting (regulate the expression of same molecule where they are located) complex molecular structures generally located at 5′-untranslated region of bacterial mRNA and respond to metabolites like coenzymes, amino acids, nucleobases, and small metal ions (Fig. 17.1). The binding of these ligands results in conformational switching which either turns on or off (attenuation of transcription or initiation of translation) the expression of mRNA. The structures and sequences of riboswitches are well conserved which also forms the basis of their classification (Grundy and Henkin 1998; Gelfand et al. 1999; Sudarsan et al. 2003a, b; Nahvi et al. 2004). Although initially discovered in bacteria, some eukaryotic riboswitches (thiamine pyrophosphate (TPP) riboswitch) were also discovered which regulates splicing (Wachter et al. 2007).

17.2 Origin and Structure of Riboswitches

Since riboswitches possess regulatory roles, it can be hypothesized that RNA world organisms possess similar structures and mechanisms to regulate gene expression. The riboswitches known today are thus the descendants from the similar structure in RNA world, for example, those that sense TPP, AdoCbl, and FMN (Barrick and Breaker 2007). Furthermore, the complexity shown by riboswitches

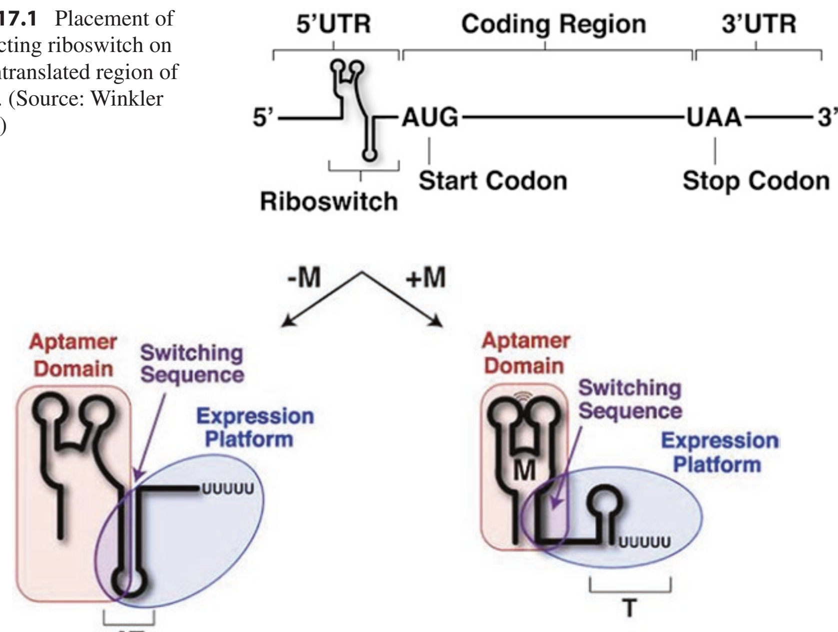

Fig. 17.1 Placement of cis-acting riboswitch on 5′-untranslated region of gene. (Source: Winkler 2005)

Fig. 17.2 Riboswitch structure containing aptamer domain, switching sequence, and expression platform. (Source: Winkler 2005)

structure must possess a long evolutionary emergence, and the molecular ligands for riboswitches are also supposed to be the remnants from RNA world (White III 1976; Benner et al. 1989).

Riboswitches can form diversified secondary and tertiary structures, but they basically assume double-stranded helical structures as found in DNA, back folding of single-stranded RNA to form hairpin structures, or the combination of these two. They are known to possess two domains, an aptamer domain which is responsible for molecular recognition and binding to the specific ligand and expression platform which is responsible for regulating gene expression in response to ligand binding by conformational switching between alternative secondary structures. In between these two domains, there exists a switching sequence, which particularly led to the conformational switching of expression platform to shut on or off the transcription (Fig. 17.2). The positioning of this switching sequence actually dictates the mRNA transcription and expression (Tucker and Breaker 2005).

17.3 Mechanisms for Riboswitch-Mediated Gene Control

Riboswitches basically implies to reversibly switch the gene or mRNA to on and off state, depending upon the ligand concentration and binding. The common mechanisms proposed for bacterial riboswitches are for transcription termination in

response to ligand binding (Barrick and Breaker 2007). The aptamer domain mostly resides in the front part of transcribing mRNA and acquires secondary structure capable of binding specific ligand (Fig. 17.1). During transcription the mRNA polymerase halts for some time after transcribing aptamer domain to allow the ligand binding. If the ligand is present in the sufficient concentration, binding occurs, resulting in the transcription termination in a Rho-independent manner. A strong stem followed by chain of uridine residues forms an intrinsic transcription terminator (Gusarov and Nudler 1999; Yarnell and Roberts 1999). The base pairing formed by this sequence further prevents the access of ribosomes to Shine-Dalgarno (SD) (ribosome binding) sequence, thereby inhibiting translation initiation. The switching sequence forms anti-terminator if the ligand is not present in required concentration, thereby allowing transcription. Due to the presence of this interplay between ligand binding, RNA folding, and rate of transcription, it was proposed that riboswitches are under kinetic control (Wickiser et al. 2005; Gilbert et al. 2006). Furthermore, the prevalence of these untranslated mRNAs is recognized by Rho protein which further accelerates transcription termination (Skordalakes and Berger 2003). The most common and widespread riboswitch class in plants and fungi (Sudarsan et al. 2003a, b) is the one regulated by coenzyme thiamin pyrophosphate (TPP) and apprehended to control splicing (Mironov et al. 2002; Winkler et al. 2002; Barrick and Breaker 2007). This riboswitch class is also present in eukaryotes with generally controlling splicing and resides in the intron region (Kubodera et al. 2003; Cheah et al. 2007; Croft et al. 2007; Bocobza et al. 2007; Wachter et al. 2007).

Another mechanism is shown by SAM-I riboswitch which responds to changing sulfur levels and controls the gene expression by being transcribed in gene opposite manner resulting in the production of antisense RNA (Rodionov et al. 2004; André et al. 2008). However, it may not be the direct sense and antisense transcript binding to control gene expression. This may be trans-acting on gene located at other places in the genome, e.g., downstream gene demonstrated in *Listeria monocytogenes* (Loh et al. 2009) and adjacent gene demonstrated in *Clostridium acetobutylicum* (André et al. 2008). The activation of ribozyme activity upon ligand binding is demonstrated for glmS class of riboswitches. Glucosamine-6-phosphate (GlcN6P) is the ligand and is the metabolic product of the protein translated by riboswitch containing mRNA. When GlcN6P concentration increases in the cell, it led to the destruction of associated mRNA coding region via activation of self-cleaving ribozymes (Winkler et al. 2004; Collins et al. 2007). The cleavage rate enhancement by activated ribozyme was shown to be more than 1 billion-fold from spontaneous RNA cleavage in *Bacillus subtilis* glmS ribozyme construct (Brooks and Hampel 2009). Other mechanisms are also proposed for the riboswitches like control of translation initiation, mRNA splicing, dual transcription, and translation controls (Skordalakes and Berger 2003).

17.4 Riboswitch Classes

The identification of riboswitches is continuously increasing with the advances in bioinformatics and molecular approaches. However on the basis of their functional characteristics and ligands, there are ten major riboswitch classes (Mehta Neel and Balaji 2010). The classification is basically done on the type of ligand (enzyme cofactor, amino acid, purine, or nucleotide base group) and the type of change in expression platform after ligand binding to aptamer domain (Ames and Breaker 2009).

17.4.1 Enzyme Cofactor Group: TPP Riboswitch

This is the most extensively studied riboswitch class predominantly present in bacteria, archaea, and eukaryotes which is known to regulate splicing and thiamine biosynthetic genes. Thiamine pyrophosphate (TPP) is the ligand which binds to the aptamer domain resulting in inducing change in expression platform and transcription termination (Serganov et al. 2006).

17.4.2 AdoCbl (Adenosine Cobalamin)

The main function is to regulate vitamin B12 (cobalamin) biosynthesis in bacteria; it is one of the first riboswitches to be discovered. Furthermore it is the largest aptamer which forms multiple contacts with its ligand adenosylcobalamin (Ado-CBL). Being cis-acting it is widely distributed in 5′-untranslated regions of vitamin B12-related genes in bacteria (Nahvi et al. 2004). The binding of Ado-CBL prevents ribosome binding and mRNA translation (Vitreschak et al. 2003).

17.4.3 SAM-I (S-Adenosylmethionine-I)

It is mostly found in Gram-positive bacteria whose structure was determined by X-ray crystallography and present on the upstream of genes involved in cysteine biosynthesis. This class of riboswitches was designated as S-box RNAs which senses SAM concentration and responds to small molecular mediators via unknown mechanism. They are proposed to regulate the translation of mRNA in cis-acting manner (Winkler et al. 2004; Montange and Batey 2006).

17.4.4 SAM-IV

Ligand is again S-adenosylmethionine (SAM) and possesses the same SAM binding site as SAM-I, but the scaffolds appear to be distinct between them. These types of riboswitches are largely present in *Actinomycetales* (Weinberg et al. 2008).

17.4.5 FMN (Flavin Mononucleotide)

The flavin mononucleotide (FMN) binding riboswitch is found in the 5′-untranslated regions of prokaryotic mRNAs which encode for protein involved in biosynthesis and transport of FMN. The mechanism of regulation is premature transcription termination (Winkler et al. 2002).

17.4.6 Amino Acid Group: Lysine

This class of riboswitches precisely senses the metabolite lysine and regulates the genes involved in lysine metabolism. Because of its lysine binding capability, it is also known as L. box. Ligand binding results in allosteric rearrangement in mRNA structure which regulate gene expression (Sudarsan et al. 2003a, b; Grundy et al. 2003).

17.4.7 Glycine

Glycine levels should be maintained in bacterial cells required for supporting protein synthesis. This class of riboswitches contains two aptamer domains with cooperative binding of glycine to each for the regulation of gene expression. This riboswitch was found to be present in the upstream of gcvT operon in *Bacillus subtilis* which regulates glycine degradation (Mandal et al. 2004).

17.4.8 Purine

In purine riboswitches the sequences of aptamer domain and expression platform are highly conserved and of about same size. The ligands are purine nucleotides guanine and adenine, binding of which are known to control both transcription and translation with diverse structures of expression platform. They are mostly known to control polycistronic mRNAs in *Bacillus subtilis* which are involved in purine nucleotide synthesis (Mandal and Breaker 2004a, b; Batey et al. 2004).

17.4.9 Nucleoside Base Group: GlmS Riboswitch

This class of riboswitches regulates the cellular concentration of glucosamine-6-phosphate (GlcN6P) by a ribozyme-containing RNA structure which controls the production of the glmS enzyme. The high levels of GlcN6P induce the ribozyme to catalyze its own cleavage leading to degradation of mRNA and thus reducing translation of glmS enzyme. The structure of this class of ribozyme was determined by X-ray crystallography (Winkler et al. 2004; Jansen et al. 2006; Hampel and Tinsley 2006).

17.4.10 PreQ1 Riboswitch (Pre-Queuosine 1)

This class contains the smallest aptamer domain as compared to other riboswitches and is known to regulate the genes involved in biosynthesis of the nucleoside queuosine. The ligand is an intermediate of queuosine biosynthetic pathway known as pre-queuosine 1 (PreQ1). It is cis-acting on downstream mRNA (Reader et al. 2004).

In addition multiple new riboswitches are being discovered with the capability to sense small molecular ligands like manganese, molybdenum, nickel, cobalt, fluorine, cobalamin, tetrahydrofolate, cyclic adenosine monophosphate (AMP), or guanosine monophosphate (GMP) with highly conserved structures. Riboswitches can also be classified on the basis of structural features into two groups known as pseudo-knotted and junctional riboswitches (Serganov and Nudler 2013). Pseudo-knotted riboswitches are those in which the RNA chain folds into a single knot-like structure from two helixes forming stem-loops, with both the loops are base paired with each other. Such structures are found in SAM-II, pre-queuosine, and fluoride riboswitches. Ligand interacts with the junction between the helices and pseudoknot groove (Gilbert et al. 2008; Klein et al. 2009; Li and Breaker 2012; Liberman et al. 2013).

Second group is known as junctional riboswitches in which multiple helical junctions form a central loop and possess several radial helices. The ligand binding site is generally located within the helical junction, but sometimes the ligand can also interact at the distant regions. The helix number is not constant with TPP and purine riboswitches containing three helices (Serganov et al. 2004, 2006) and flavin mononucleotide (FNM) riboswitch containing six helices (Serganov and Patel 2009).

17.5 Riboswitches as Potential Antimicrobial Drug Targets

Riboswitches' ability to recognize the specific ligands makes them an ideal candidate for antibacterial drug therapy. The bacterial resistance against antibiotics is progressively increasing which increases the demand of potential new antibiotics. The ligand mimetics for riboswitches have the potential to be used as antibiotics and possess many advantages over traditional antibiotics (Fig. 17.3). Firstly, as

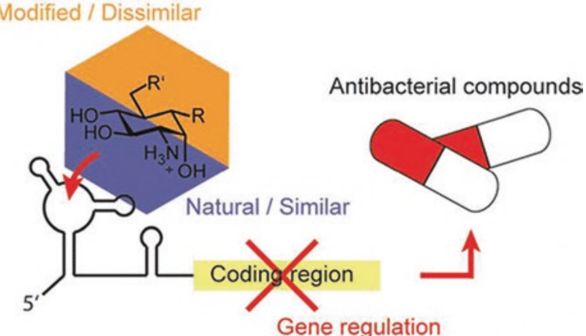

Fig. 17.3 Action of anti-bacterial compounds/drugs which are ligand mimetics/analogues of bacterial riboswitches. (Source: Matzner and Mayer, 2015)
Being similar in structure, these ligand mimetics can bind the aptamer domain but will not led to conformational switching and thus inhibits the translation of the downstream gene which regulates the metabolite concentration for bacterial growth and division. These ligand mimetics thus are promising candidates for antibacterial drug discovery process. (Source: Matzner and Mayer 2015)

riboswitches are predominantly present in prokaryotes, they are having lower toxicity to higher eukaryotes, including humans. Secondly, their specificity to recognize small molecular metabolites makes them easy to regulate and deliver. Thirdly, the bacteria cannot easily develop resistance against riboswitches as there are multiple copies on multiple genes, so a single mutation is insufficient to develop resistance.

Purine riboswitches like 2′-deoksyguanosine, adenine, guanine, and prequeuosine (preQ1) controls the metabolism and transport of purines (Lünse et al. 2014). They possess the conserved aptamer domain with the difference being of only one nucleotide which can differentiate between ligands. For example, in adenine and guanine, there is a difference of only one nucleotide, i.e., pyrimidine at the 74th position which forms Watson-Crick interactions with the ligand (Gilbert et al. 2009). As purines are very essential for the actively dividing bacterial cells, purine analogs like 6-N-hydroxylaminopurine can be used to regulate these riboswitches and thus bacterial growth (Kim et al. 2007).

Lysine riboswitch with 200 nucleotide length and five stem-loop structures is one of the largest and most complex riboswitches (Sudarsan et al. 2003a, b). Many lysine analogs like L-aminoethyl cysteine (AEC), L-4-oxalysine, L-3-[(2-aminoethyl)-sulfonyl]-alanine, and Dl-trans-2, 6-diamino-4-hexenoic acid can be used as potential antimicrobial compounds (Ataide et al. 2007; Blount et al. 2007). Being similar to lysine in structure, these analogs bind effectively to lysine riboswitch leading to gene repression and less lysine production in bacterial cell. It was found that using these lysine analogs, the bacterial concentration had been fivefold inhibited in 6 h and completely inhibited in 24 h. However, AEC can incorporate into proteins in mammalian cells, leading to toxicity (Di Girolamo et al. 1986, 1990). AEC can also bind lysyl-tRNA synthetase (LysRS) which can increase bacterial lysine production and thus resistance to AEC (Ataide et al. 2007).

The prokaryotic major secondary messenger cyclic-di-GMP controls various functions in bacteria like adhesion, biofilm formation, cell aggregation, and virulence. The two classes of riboswitches sense the 3′-endo conformation of ribose to specifically bind cyclic-di-GMP but respond with different mechanisms. Class-I follows the transcription termination mechanism upon ligand binding (Sudarsan et al. 2008), whereas Class-II follows the allosteric formation of self-splicing ribozymes (Lee et al. 2010). Because of its absence in eukaryotic organisms, it's an ideal candidate for antibacterial drug therapy (Chen and Schaap 2012). Analogs to cyclic-di-GMP have also exploited for designing anti-biofilm agents to prevent the bacterial biofilm formation responsible for causing many diseases and contaminating equipment (Reyes-Darias and Krell 2017).

The glmS gene, present in Gram-positive bacteria, is important to synthesize glucosamine-6-phosphate (GlcN6P) which is a precursor of peptidoglycan for biosynthesis of bacterial cell wall (Collins et al. 2007). As the product of this gene is important for bacterial division, which is regulated by ribozyme riboswitch in the 5′ upstream region, effort has been made to identify the ligand analogs. It was identified that phosphate group in the ligand is essential for high-affinity binding, whereas amine group is important to regulate the activity of riboswitch (Winkler et al. 2004; McCown et al. 2011).

Thiamine pyrophosphate (TPP) is a crucial cofactor for metabolism of amino acid and sugar. The TPP riboswitch recognizes ligand by its interaction with both pyrophosphate and pyrimidine of TPP (Ontiveros-Palacios et al. 2008; Thore et al. 2006). Pyrithiamine (PT) is the first described analog of TPP to demonstrate antibacterial properties (Sudarsan et al. 2005). But the reports have shown the mechanism by which the bacteria acquire resistance by accumulating mutations in aptamer domain which degrades this riboswitch. However, there are other TPP analogs which show potential binding and transcription termination through TPP riboswitch, for example, benfotiamine (Edwards and Ferre-D'Amare 2006), 4-methyl-5-hydroxyethylthiazole (Winkler et al. 2002), amprolium, and oxythiamine (Thore et al. 2008).

FMN also plays an essential role in fats, carbohydrates, and protein metabolism whose concentration is regulated by FMN riboswitch. The phosphate residue on FMN is involved in binding to two peripheral stem-loops in the aptamer domain resulting in gene repression (Serganov and Patel 2009; Ott et al. 2009). Roseoflavin, an analog of riboflavin, is a natural antibiotic produced by bacteria *Streptomyces davawensis* which is converted into roseoflavin-5-monophosphate (RoFMN), binds FMN riboswitch, and inhibits the expression of downstream genes (Lee et al. 2009; Ott et al. 2009). It possesses higher binding affinity than native FMN, but research had shown that a nucleotide in aptamer domain at position 61 is responsible for discrimination of the ligands (Pedrolli et al. 2012). Another natural riboflavin analog 8-demethyl-8-aminoriboflavin (AF), produced by the bacteria *Streptomyces cinnabarinus*, also acts in a similar way (Matern et al. 2016).

Recently ribocil-C was identified as a very selective inhibitor of FMN riboswitch and controls the expression of riboflavin/vitamin B2 biosynthesis in *E. coli*. It was demonstrated that FMN riboswitches perform dual functions of riboflavin

Table 17.1 Ligand analogs which show antibacterial property

S. No.	Ligand	Analog	Riboswitch	References
1.	Purine	2′-Deoksyguanosine	Purine riboswitch	Lünse et al. (2014)
2.	Purine	6-N-Hydroxylaminopurine	Purine riboswitch	Kim et al. (2007)
3.	Lysine	L-Aminoethyl cysteine (AEC)	Lysine riboswitch	Ataide et al. (2007)
4.	Lysine	L-4-Oxalysine	Lysine riboswitch	Blount et al. (2007)
5.	Lysine	L-3-[(2-Aminoethyl)-sulfonyl]-alanine	Lysine riboswitch	Blount et al. (2007)
6.	Lysine	Dl-trans-2, 6-Diamino-4-hexenoic acid	Lysine riboswitch	Blount et al. (2007)
7.	Thiamine pyrophosphate (TPP)	Benfotiamine	TPP riboswitch	Edwards and Ferre-D'Amare (2006)
8.	Thiamine pyrophosphate (TPP)	4-Methyl-5-hydroxyethylthiazole	TPP riboswitch	Winkler et al. (2002)
9.	Thiamine pyrophosphate (TPP)	Amprolium	TPP riboswitch	Thore et al. (2008)
10.	Thiamine pyrophosphate (TPP)	Oxythiamine	TPP riboswitch	Thore et al. (2008)
11.	Riboflavin	Roseoflavin	FMN riboswitch	Lee et al. (2009) and Ott et al. (2009)
12.	Riboflavin	8-Demethyl-8-aminoriboflavin (AF)	FMN riboswitch	Matern et al. (2016)
13.	Riboflavin	Ribocil-C	FMN riboswitch	Wang et al. (2017)
14.	Flavin	5-(3-(4-Fluorophenyl)butyl)-7,8-dimethylpyrido[3,4-b]quinoxaline-1,3(2H,5H)-dione (5FDQD)	FMN riboswitch	Blount et al. (2015)

biosynthesis and its uptake necessary for methicillin-resistant *Staphylococcus aureus* (MRSA) growth and pathogenesis. Ribocil-C, being capable of inhibiting both activities, thus is a broad-spectrum antibiotic against Gram-positive bacteria (Wang et al. 2017). A synthetic flavin analog 5-(3-(4-fluorophenyl)butyl)-7,8-dimethylpyrido[3,4-b]quinoxaline-1,3(2H,5H)-dione (5FDQD) had also been designed to bind FMN riboswitch with equal potency and thus shows antibacterial activity to protect mice from *Clostridium difficile* infection (Blount et al. 2015). This information about riboswitches ligand analogs is summarized in Table 17.1.

17.6 Riboswitches as Biosensors

An analytical device which combines the specificity of biological system with physicochemical detector to detect an analyte is known as biosensor. A number of biological compounds like enzymes, antibodies, proteins, receptors, DNA, RNA, etc. which harbor the property of specificity and can be recognized were used to develop biosensors (Han et al. 2010; Arora 2013). Most commonly used biosensors like glucometer generally employ immobilized enzymes which when bound to substrate undergo a biochemical reaction resulting in a release of electrochemical signal identified/quantified by detector (Arora 2013; Su et al. 2011). Since the production of purified enzymes accommodates high cost, efforts have been focused to develop biosensors with low cost but improved sensitivity and stability which were having whole cells immobilized on supporting material (Park et al. 2013).

The sensitivity and specificity of the riboswitches for their ligands are now being exploited for the development of new biosensors. The fusion of the reporter protein to the riboswitch is utilized to create intracellular biosensor which can detect even small concentration of molecular ligands. A biosensing man-made riboswitch was developed which uses hepatitis delta virus ribozymes to cleave virus RNA transcripts after recognizing specific ligand (Bergeron and Perreault 2005). The name "aptazyme" was given to such fusions of an aptamer domain recognizing specific ligands to a self-cleaving ribozyme (Rehm et al. 2015). A riboswitch-based colorimetric biosensor had been developed for the detection of glucosamine-6-phosphate (GlcN6P), a drug target of many antibiotics. The binding of GlcN6P led to self-cleavage of glmS ribozyme which releases a RNA fragment and signals/amplifies the release of colorimetric reporters, G-quadruplex DNAzymes (Zhao et al. 2014). Further, a light-sensing riboswitch which control gene expression had also been designed using close isomers of dihydropyrene (Sen 2008). An artificial riboswitch which responds to photon of light had been constructed by using a photo-responsive ligand which contains azobenzene and when irradiated with light undergoes photo-isomerization (Hayashi and Nakatani 2014).

Computational methods were effectively utilized to improve the biochemical designing of riboswitches. Computerized simulations with energy minimization procedures were first used to identify the sequences of aptamer domain and expression platform with required characteristics (Avihoo et al. 2007). The sequence of aptamer domain which can bind specifically to a particular ligand can be de novo generated by in vitro selection process called systematic evolution of ligands by exponential enrichment (SELEX). The SELEX allows the generation of synthetic riboswitches which can recognize any small molecule in low concentrations (Mayer and Famulok 2009). The first riboswitch biosensor to be developed was theophylline biosensor in 2009, which monitor the concentration of an antiasthmatic drug, theophylline (Jo and Shin 2009). High concentrations of theophylline cause many health problems like heart rhythm disturbances and seizures (Pernites et al. 2011). So to monitor this, aptamer domain specific to theophylline was generated. Linking this aptamer domain to GFP coding sequence on 3′ end led to dose-dependent detection of theophylline, translated into induction of GFP expression which is

shown as digital data by signal transducer. Further, using riboswitches, the concept of programmed evolution arises which enables scientists to program bacteria using specific ligands and use evolutionary phenomena to check the genetic control elements which regulate metabolism (Eckdahl et al. 2015).

The rational design strategy was also used to design riboswitch biosensor which senses small molecular ligands and work in cell-free translation system (Ogawa 2011). It was demonstrated that aptamer binding to ligand follows charge transfer kinetics and charge conductivity in nano-ampere range which further increases the applications of biosensors (Schill and Koslowski 2013). A fluorescent biosensor had been developed which can be used for live cell imaging. This RNA-based biosensor was designed in vivo by fusing the natural GEMM-I riboswitch with spinach aptamer which confirm the production of cyclic GMP/AMP as a fluorescent signal by turning on and off (Kellenberger et al. 2013). Further, the electrophoretic mobility shift assay (EMSA) was developed by using aptamer domain of Vc2 riboswitch which can be used to quantify the concentration of cyclic GMP (Kellenberger et al. 2015). The synthesis of position-specific labeled RNA had been automated using modified riboswitches and robotic platform which proves useful for studying RNA dynamics and structure, making RNA sensors and cell biological studies (Liu et al. 2015).

Recently a kissing complexes-induced aptasensor (KCIA) had been developed to analyze adenosine and adenosine deaminase (ADA). This aptasensor integrates silver nanoclusters (AgNCs) with adenosine riboswitches which upon ligand binding stabilizes AgNCs with nucleic acids and is thus detected (Zhang et al. 2016). A cGAMP synthase (cGAS) RNA-based florescent biosensor had been developed to detect and quantitate cyclic GMP/AMP secondary messengers in mammalian cells which plays an important role in regulating immune responses (Bose et al. 2016). Recently a synthetic theophylline riboswitch had been synthesized which was labeled with dual color reporter whose color was determined by the presence or absence of a particular ligand. The reporter uses *E. coli* fimbriae phase variation system and was composed of genes for two fluorescent proteins GFPa1 (green) and mKate2 (red). A constitutively active promoter was placed between these two genes such that in the absence of ligand GFPa1 was constitutively expressed imparting green color, but when the ligand was present, there was a translational inversion which led the constitutive expression of mKate2 imparting red color (Harbaugh et al. 2017). A naringenin-responsive riboswitch had also been synthesized which contain this fluorescent dual reporter to identify naringenin-producing *E. coli* in coculture (Xiu et al. 2017).

17.7 Conclusions

Although riboswitches have been discovered two decades ago, newer riboswitches with different ligand specificities are being discovered every year showing the interest of the scientific society in riboswitches. Natural riboswitches, thought to be reminiscent of the RNA world, control many important functions in the prokaryotic

cells particularly required in the absence of the other regulatory mechanism like epigenetic histone modifications and chromatin dynamics. These riboswitches can effectively sense the presence/absence of small molecular ligands and regulate the expression or repression of genes responsible for formation/metabolism of that ligand. The sensitivity and specificity of riboswitches for their ligands can be harnessed for the synthesis of synthetic riboswitches.

Additionally their important role in regulating the concentration of particularly those ligands which are essential for bacterial survival and growth can be used to design antibacterial drugs. Many naturally occurring microorganisms are already using this phenomenon to inhibit the growth of unwanted bacteria by secreting ligand analogs for specific riboswitches. The development of synthetic analogs of riboswitch ligands further broadens their application for the synthesis/discovery of antibacterial drugs. Although these synthetic ligand analogs are proven to be efficiently controlling the growth of bacteria, but till date none of them have reached the clinical trials. The reason may be their weak binding with the aptamer domain which could not match the binding of natural ligand or the potential of bacteria to easily acquire resistance against these analogs as they only control the expression of single gene.

Furthermore, the fusion of the ligand binding aptamer domain of riboswitch with the downstream reporter gene controls the expression of reporter gene primarily governed by the presence or absence of ligands. This concept was utilized for the construction of in vivo and in vitro biosensors used to either monitor the bacterial behavior in response to wide variety of ligands or control the bacterial gene expression by regulating ligand concentration. Multiple reporter genes are used for the construction of biosensors like green fluorescent protein, silver nanocluster, and dual color reporter system. The advent of the SELEX programs led to the development of aptamers to a wide range of ligands which further increases the applications of riboswitches. The concept of programmed evolution, which automated the control of riboswitches by using specific ligands, increases their application to bioremediation, biomining, energy conversion, and pharmaceuticals.

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18 Microbial Metagenomics for Industrial and Environmental Bioprospecting: The Unknown Envoy

Daljeet Singh Dhanjal and Deepansh Sharma

Abstract

Microorganisms grown as laboratory strains have been the starting approach for the discovery of various industrially viable molecules now in use. Metagenomics, which exploits culture-independent approach to access the combined genomes of environmental microbial populations, offers a resource of exploring the microbial metabolites derived from the large pool of microorganisms that are known to exist in the environment but remain obstinate to laboratory culturing. Highly specific, tailor-made, novel microbial metabolites have been obtained using metagenomic methods for industrial and environmental sustainability. The application of microbial metagenomics is not limited to the population ecology but can also have huge scope for pharmaceutical and environmental sustainability. Thus, now the analysis in the real time about the expression of the particular gene is to be studied for the activity, efficacy, consistency and specificity. Combined exertions linking researchers from diverse fields comprising microbial genetics, genomics, bioinformatics and synthetic biology will be expected to be essential to the commendable potential of these unknown envoys for a sustainable future. The current developments in microbial metagenomics to the discovery of industrial important molecules will be discussed in the current book chapter.

Keywords

Bioprospecting · Industrial enzyme · Metagenomics · Metabolites · Microbial diversity

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18.1 Introduction

Earth is the niche of >10^{30} microbes which is nine times to the number of stars known to humanity (Knight et al. 2012). As we all are aware, life initiated from these single-cell microbes, and with evolution it has evolved and assimilated itself to survive which we can visualise by the vast diversity around us. However, these tiny little creatures are not only predominantly driving the functioning of the ecosystem but are continuously evolving (Nannipieri et al. 2003). That is why a still large and interdependent number of these microbes are unknown by approximately 99% (Kaeberlein et al. 2002). The traditional culturing approach was limited to the growth of bacteria which were compatible to catabolise the known culturing media and kept us forbidden from the microbes that owe undefined growth conditions. The conventional approach was named as 'metagenomics', in which the genetic material is isolated directly from the environmental sample, amplified and sequenced using 16S ribosomal RNA. This approach enabled us to gather extensive information regarding the novel bacterial species (Ferrer et al. 2005a, b, c; Piel 2011; Liebl et al. 2014).

Metagenomics is also known as environmental or community genomics as it is the blend of bioinformatics, genomics and system biology. In 1998, Jo Handelsman and his colleagues used the 'metagenomics' term for the first time and stated it as the technique in which cloning, as well as analysis of functionality, is done on genome microbial community (Handelsman et al. 1998). The certain modification has been done to the definition of the metagenomics which stated it to be the modern technique of genomics which allows us to study and analyse the microbial community directly from their natural ecosystem. The metagenomic emanation has facilitated us to overcome the drawback of the traditional approach and lets us infer the knowledge about the real microbial diversity which was previously masked. This uncultured diversity of microbes is the hub and continuous source of novel metabolites which may be of industrial importance (Chen and Pachter 2005). These 99% uncultured microbes leave us a fascination of what they might hold within themselves as less information regarding their genes, genome and enzyme functionality is known (Kaeberlein et al. 2002).

The advent of metagenomics not only complements but surpasses the traditional culturable approach and its limitations. The research employing this approach provided the positive feedback as it has enabled the researcher to explore and understand microbes to more depth as well as in identifying novel metabolites (Culligan et al. 2014). The metagenomic libraries are constructed for storing and analysing genetic information of the isolated microbes from the environmental sources. Thus gathered information enables us to explore about the microbes present, the function of microbes and the potential of microbe genetic material for the welfare of mankind. Further, this approach shows the similar working mechanism of the approaches such as metatranscriptomics and metaproteomics which are also used for exploring functionality. Metagenomics is a robust tool which stimulates original postulates associated with the function of microbes; the remarkable discovery of novel human viruses and novel antibiotics testifies to this fact (de Vos and de Vos 2012).

The crude and unprocessed extract of microbes encloses various novel metabolites of different configuration. Thus, the exertion for evaluating these active metabolites of biological origin is defined as bioprospecting (Lahlou 2013). The main function on which metagenomics is based is to excavate the genes which are responsible for encoding the novel metabolite. The possibility for identifying the novel metabolites majorly depends on the number of strains isolated with the addition to the diversity among them and their metabolite synthesising mechanism which makes it unique. Due to such convolution in the metagenomic sample, high-efficiency and sensitive screening techniques are required that enable us to produce rapid and reliable results for identifying genes encoding for novel metabolite from the pool of metagenomic library which is constructed. Thus, before exploring the novel metabolites, thorough processing of all aspects is done (Sharma and Vakhlu 2014).

18.2 Excavation of Metagenomes for Novel Metabolites

For mining the novel metabolites from an environmental sample, there is a major need for the selection of the sampling site. After the selection of the sampling environment, the process of DNA extraction, amplification of the extracted DNA and construction of metagenomic library gets started. After the construction of the metagenomic library, the analysis of data obtained is done by two approaches: (a) screening based on sequences and (b) screening based on function. Both the approaches follow certain sets of approaches for generating the sequence. The sequence obtained is thus analysed using the different bioinformatic tools (Thomas et al. 2012). The workflow of the metagenomic for mining of novel metabolites is illustrated in Fig. 18.1:

18.3 Sampling from Various Environmental Sites

Microbes are omnipresent on this Earth; either their origin is biotic based or abiotic based. These two habitats differ from each other as abiotic refers to air, soil and water (either marine or freshwater), whereas biotic refers to microbiota of animals, insects, plants or other living organisms (Knight et al. 2012). Current investigations now focus on identifying the microbial diversity from the unexplored locations in the environment to get the better insight of microbial species up to their genetic and metabolic level. Nowadays, metagenomic examination is conducted by taking sampling sites in consideration and is broadly separated into three categories (Steele et al. 2009):

18.3.1 Category 1

Sampling is done from a diverse location such as soil or marine water. In this category, the DNA is directly isolated from the environmental sample or the sample supplemented with nutrient to enrich the number of microbes which synthesise the desired metabolite.

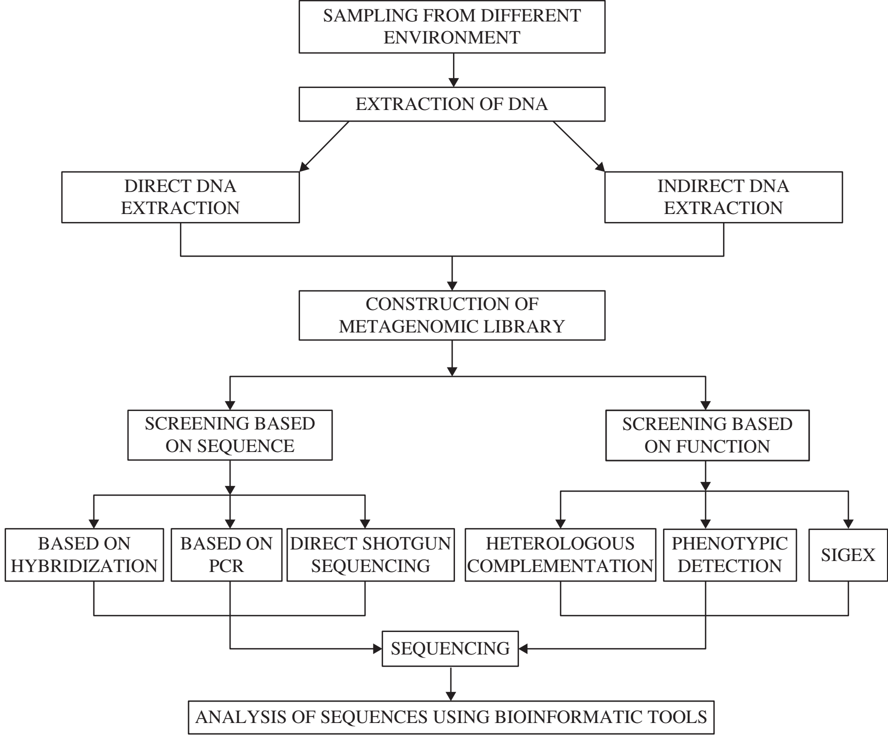


Fig. 18.1 Workflow of metagenomics for mining of novel metabolites (Adapted from Sharma and Vakhlu 2014)

18.3.2 Category 2

Sampling is done from the natural ecological niche of the targeted metabolite like xylanase which is present in the gut of insects.

18.3.3 Category 3

Sampling is done from the extreme environment as these microbes have the ability to synthesise the metabolites in their active state under adverse conditions.

18.4 Extraction of DNA

The procedure followed for isolating the metagenomic DNA from the diverse environment can be classified into two categories: direct DNA extraction and indirect DNA extraction. Microbial diversity as the name implies is the concoction of microbes, which have a different composition of the cell wall that alters the

vulnerability to lysis. Thus, this ensures that there is a need to develop isolation procedures so that bacterial cell wall can be lysed in a sample and their genome gets extracted simultaneously. Though on extracting the metagenomic DNA, all the type of the cells present in the sample should get extracted and can be altered up to the molecular level (Daniel 2005). For the prosperous result, during the extraction of the metagenomic DNA from a diverse environment, many different isolation procedures have been developed, whereas many of these procedures are now available in the form of extraction kits. These extraction kits vary due to their purity as well as inhibitors required for the successive DNA isolation. All the procedures for isolating metagenomic DNA comprise of chemicals, detergents and enzymes. Most commonly, lysozyme and SDS (sodium dodecyl sulphate) are used for disruption of the cell wall, whereas some procedures involve the implication of mechanical forces for lysis of cell wall such as beating with beads, thawing after freezing or sonication. For assessing the maximum microbial diversity, it is recommended to use more than one isolating procedure for extracting metagenomic DNA. Thus, two main categories for the extraction of metagenomic DNA are direct DNA extraction and indirect DNA extraction (Kimura 2006; Purohit and Singh 2009).

18.4.1 Direct DNA Extraction

This method involves the lysis of cell with sample matrix after lysis DNA is separated from the matrix as well as cell debris (Sharma and Vakhlu 2014). Different direct extraction methods have been compiled in Table 18.1.

18.4.2 Indirect DNA Extraction

This method involves the prior separation of the cells from the soil matrix. After that, the cell is lysed, and DNA is extracted (Sharma and Vakhlu 2014). Different indirect extraction methods have been compiled in Table 18.1.

18.5 Construction of Metagenomic Library

After isolating the DNA, the next steps involve the construction of the metagenomic library. For constructing the library, the metagenomic DNA is fragmented and is cloned into the particular vector. The specific vector is then inserted into the host strain where the screening of the gene or function of the gene is studied. Generally, the metagenomic library is constructed for the large fragments of DNA which are about 25–200 Kb, and these stretches of DNA are inserted into specific vectors. The vectors are selected on the basis of the DNA size which is to be cloned. DNA fragment of size 100–200 Kb is inserted in bacterial artificial chromosome (BAC) used, 25–35 Kb in cosmids, 25–40 Kb in fosmids and over 40 Kb in yeast artificial chromosome (YAC). Thus, on the basis of the size of inserts, two broad categories for

Table 18.1 List of different methods with their procedures for extraction with advantages and disadvantages

Methods	Cell lysis	DNA purification	Advantages	Disadvantages	References
Direct DNA extraction					
Ogram method	Beads are used to break the cells. Incubation with SDS at 70 °C	DNA precipitation with PEG and PEG extraction with phenol-chloroform. Density gradient with caesium chloride. DNA concentration and purification using ethidium bromide	Higher yield of DNA per gram of sediment. Ideal for procedures of direct hybridisation DNA-DNA	Time-consuming method. Very fragmented DNA	Ogram et al. (1987)
Moran method	Use of lysozyme, followed by freezing and thawing to lyse the cells	Phenol-chloroform extraction. DNA precipitation with isopropanol. Removal of impurities by molecular exclusion in a gel permeation column	A fast methodology, obtaining less fragmented DNA. Recommended for Southern blotting due to the lower DNA fragmentation	The DNA obtained contains many impurities but with less contaminating eukaryotic DNA	Moran et al. (1993)
Zhou lysis method	Use of lysis buffer, lysozyme, proteinase K and SDS, with steps of re-extraction	Extraction with chloroform and precipitation with isopropanol, followed by washes with 70% ethanol. DNA purification by recovering it after electrophoresis on a 3% agarose gel	Large quantities of total DNA recovered	Low diversity	Zhou et al. (1996)
Harsh lysis method	Use of zirconia/silica beads, followed by vortexing for lysis of the material prepared as in the soft lysis method	Extraction with chloroform and precipitation with isopropanol, followed by washes with 70% ethanol. DNA purification by recovering it after electrophoresis on a 3% agarose gel	Method faster and easier than the soft lysis method	Low diversity and highly fragmented DNA	Gabor et al. (2003)

Indirect DNA extraction					
Holben blending method	Obtaining the sample cells by successive steps of dilution in a specific mixer buffer followed by low-speed and then high-speed centrifugation. Cell lysis using lysozyme and proteinase K, following the protocol of the direct soft lysis method	Extraction with chloroform and precipitation with isopropanol, followed by washes with 70% ethanol. DNA purification by recovering it after electrophoresis on a 3% agarose gel	Best recovery of prokaryotic cells with great diversity. Less fragmented DNA	Recovery of low concentrations of total DNA	Holben et al. (1988)
Jacobsen and Rasmussen method	Harvesting of cells by cation exchange resin. The cells obtained are treated with lysozyme and pronase	Density gradient of caesium chloride, followed by ethidium bromide for DNA purification	Purer and less fragmented DNA	Time-consuming (4 days of extraction) and somewhat expensive	Jacobsen and Rasmussen (1992)

library construction are formed in which 15 Kb of fragmented DNA is inserted into the plasmid, whereas the large stretch of DNA is inserted into vectors such as cosmids, fosmids, BAC and YAC. The advantage of the small-insert library over large-insert library is that lysis for the isolating DNA can be executed by a harsh procedure where shearing of DNA occurs during extraction (Riesenfeld et al. 2004a, b).

The approach for constructing library changes in accordance with the target of the study. It is recommended that DNA inserts for studying the gene and its involvement in metabolic pathways should be cloned in the vector of high molecular weight. The vector with high molecular weight increases the chances of positive result during the screening process, whereas if the small vectors are used, then more number of cloning vectors will be required for metagenomic analysis of the full sequence (Green and Keller 2006). There are many other cases where an expression of the particular gene is not executed on the single host. In that cases, the broad range of hosts (*Bacillus*, *Streptomyces* and *Pseudomonas*) are used to study the expression of the gene (Courtois et al. 2003; Martinez et al. 2004; Lorenz and Eck 2005). This approach provides the positive results as the frequency of gene detection involving unique function increases. Plasmid RK2 is one of the broad-range vectors (Aakvik et al. 2009).

18.6 Metagenomic Data Screening

This section focusses on the two approaches for analysing the data obtained after the construction of the metagenomic library. Screening of the data is executed either on the basis of sequence or on the basis of the function performed by antibiotic-resistant gene or enzyme.

18.6.1 Based on Sequence

Sequenced-based screening may work on various aims, extending from analysing of microbial diversity of the target environment, isolation of novel viruses (Vieites et al. 2009), investigating of the novel catabolic gene, investigation of the mobile element existing in the gene of bacteria or phylogenetic reconstruction by analysing the genes of ancestral microbial species (Kunin et al. 2005; Jacquiod et al. 2014).

The different methods employed for the sequence-based screening comprise of the approaches based on hybridisation, PCR and direct shotgun sequencing.

18.6.1.1 Screening Based on Hybridisation

Screening based on hybridisation involves the construction of probes of the homologous sequences already present in the databases available online. These homologous sequences used for synthesising probes are target genes which encode for a particular enzyme such as chitinase, dioxygenase, hydrogenase, reductase, oxidoreductase, etc. This enables us to find the enzyme involved in degradation of pollutants, genes contributing in the antibiotic synthesis and identifying the new species

of the taxonomic group (Jacquiod et al. 2014). Nowadays, this hybridisation-based analysis is done by using microarray, as these chips comprise of the probes restricted to different genes. Some of the commercially available microarray chips are Chip for antibiotic-resistant gene, GeoChip, HuGChip, HITChip and Virochip (Miller and Tang 2009; Tu et al. 2014).

18.6.1.2 Screening Based on PCR

The initial proceedings for analysing microbial biosphere changed our opinion, as rRNA played the role of evolutionary biomarker and progression of the PCR (Simon and Daniel 2011). As the screening based on PCR can be targeted to analyse the microbial community for the gene encoding for antibiotic, enzyme and antibiotic resistance. This is the most common approach which is employed for screening the metagenomic data and finding the phylogenetic relationship among the species. Some reports even stated that primers used in this approach help in synthesising probes for the detection of catabolic genes during hybridisation screening (Sharkey et al. 2004; de Castro et al. 2014).

18.6.1.3 Screening Based on Direct Shotgun Sequencing

Due to the advancement in sequencing techniques, DNA obtained from the complete metagenomic clone or environmental sample is sequenced entirely so that diversity among the microbial community can be assessed (Thomas et al. 2012). This approach of analysis has generated the massive amount of data for the assessment at very low cost and enables us to analyse the sequence for functional as well as taxonomic diversity (Kim et al. 2013). The information of the sequence obtained from metagenomic library assists in primer and probe designing which is specific for cloning the gene of interest. The screening of environmental sample by the direct shotgun sequencing enables us to identify the novel gene and organism (Vieites et al. 2009).

18.6.2 Based on Function

Uncultured microbes are thought to be the reservoir of the novel metabolites. They are yet to be explored due to which potential of these microbes remains hidden. This approach facilitates in identifying the novel genes which encode the novel metabolites as their metabolic activity is screened in constructed cloned metagenomic library (Riesenfeld et al. 2004a). It is the only approach which enables us to identify the novel class of genes which encode for known or new function as it is independent of the previously collected information about the gene function or similarity. The effectiveness of function-based screening is more as it involves various parameters. The parameters which play a crucial role comprise the host organism, vector, target gene size as well as its abundance in the metagenomic sample, a method of assessment and its efficacy in the surrogate host for its heterologous gene expression. Thus, for the evaluation of metagenomic library, three different approaches are used (Riesenfeld et al. 2004b; Ferrer et al. 2009).

18.6.2.1 Heterologous Complementation

It is one of the most frequent and rapid approach, in which the clone is assessed and identified for expressing the desired function. The principle behind this approach is heterologous complementation among the host strain or its mutant which requires the presence of target gene for the growth under controlled conditions. Recombinant clones are expressed as they contain the target gene which keeps them active and allows it to grow in controlled conditions. Due to selective nature of this process, no false-positive result is generated by this approach making it one of the efficient and suitable methods (Simon and Daniel 2011).

18.6.2.2 Phenotypic Detection

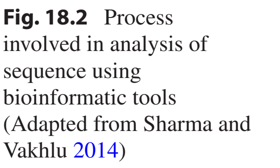
In this method, dyes and enzyme substrate derivatives containing chromophore are present in the growth medium. Due to the presence of the target sequence or metabolite, they grow in the growth medium and cause phenotypic change that can be visualised. Thus, the working of the metabolic functioning of the clone is recorded (Gloux et al. 2011).

18.6.2.3 SIGEX

SIGEX, also known as substrate-induced gene expression, is the technique developed in 2005 by Uchiyama and his colleagues. The principle behind this method is established on the fact that for the functioning of the catabolic gene, it needs external stimuli for its expression (Uchiyama et al. 2005). Usually, the regulatory sequence is present nearby the genes which are needed to be expressed. SIGEX helps in the inspection of clones embraced with a catabolic gene which expresses when the substrate is present. The major advantage of this method is semiautomated which saves time, workforce and other expenditures. It is stated to the high-throughput method as it employs FACS, which facilitates in rapid cloning of different gene in a short period of time. The main reason for this method of interest for function-based screening is it doesn't need any toxic or expensive substrate to detect the catabolic genes (Lorenz and Eck 2005; Uchiyama and Watanabe 2007).

18.7 Analysis of Sequences Using Bioinformatic Tools

The most important step of the metagenomic is the analysis of the data retrieved from the environmental sample with more complexes in comparison to the previously generated data. The data of metagenomic sequences is less redundant and in large amount due to a small stretch of DNA quality of the sequence is low, whereas polymorphic rate is high. The data is growing at such pace as it has moved from megabase to terabase pairs, which now need a high-throughput computational system and expert for the analysis. Different approaches have been developed which employ computational technology and analyse the raw data. The result acquired on analysis is mainly dependent on the approach taken as each approach has its own pros and cons. Thus, the evolution of the bioinformatic tools is taking place for the



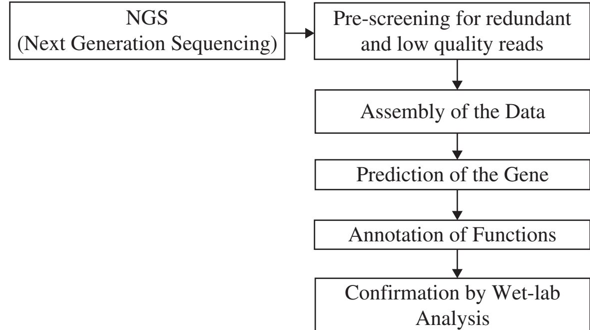


Fig. 18.2 Process involved in analysis of sequence using bioinformatic tools (Adapted from Sharma and Vakhlu 2014)

precise metagenomic analysis (Sharma and Vakhlu 2014). Figure 18.2 illustrates the process involved during analysis of sequence using the bioinformatic tool.

Thus, the metagenomic approach has enabled us to update the tree of life by the phylogenetic analysis and assessing the diversity among microbes illustrated in Fig. 18.3.

18.8 Applications of Metagenomics

18.8.1 Environmental Application

Due to the development of the industrial sector, the new pollutants have emerged in the environment. These toxic and artificially synthesised compounds have forged microbial community to survive in these conditions. Many adverse effects produced by these compounds have been documented. To antagonise these effects, the environment-friendly and economical methods of remediation are being explored to eradicate the pollutant with the help of these microbes.

Biodegradation is one of the processes which involves the microbes for the breakdown of complex compounds in association with abiotic as well as biotic entities so that they get to blend into the biogeochemical cycle. In general, biodegradation means the conversion of the complex organic compound into simple compounds with the help of microbes. Microbial bioremediation has emerged as an effective strategy for eradicating anthropogenic entities from the contaminated environment. This is where metagenomics comes into the picture, as it is the only strategy to unexplore the bacterial community present in the contaminated site and possess the ability to catabolise the toxic compounds. Thus, there is a need for the identification of the genes that play a crucial role in the catabolising the toxic compound or discover the pristine metabolic pathway that can convert the pollutant into mineral which would get admiration in bioremediation and industrial field. The traditional approach for remediation (such as incineration, landfilling, etc.) was expensive and not 100% effective. On the other hand, the bioremediation approach for treating the industrial contamination requires prior knowledge about the growth factors, metabolism, dynamics and functions of native microbial community present at the contaminated site (Gupta and Sharma 2011).

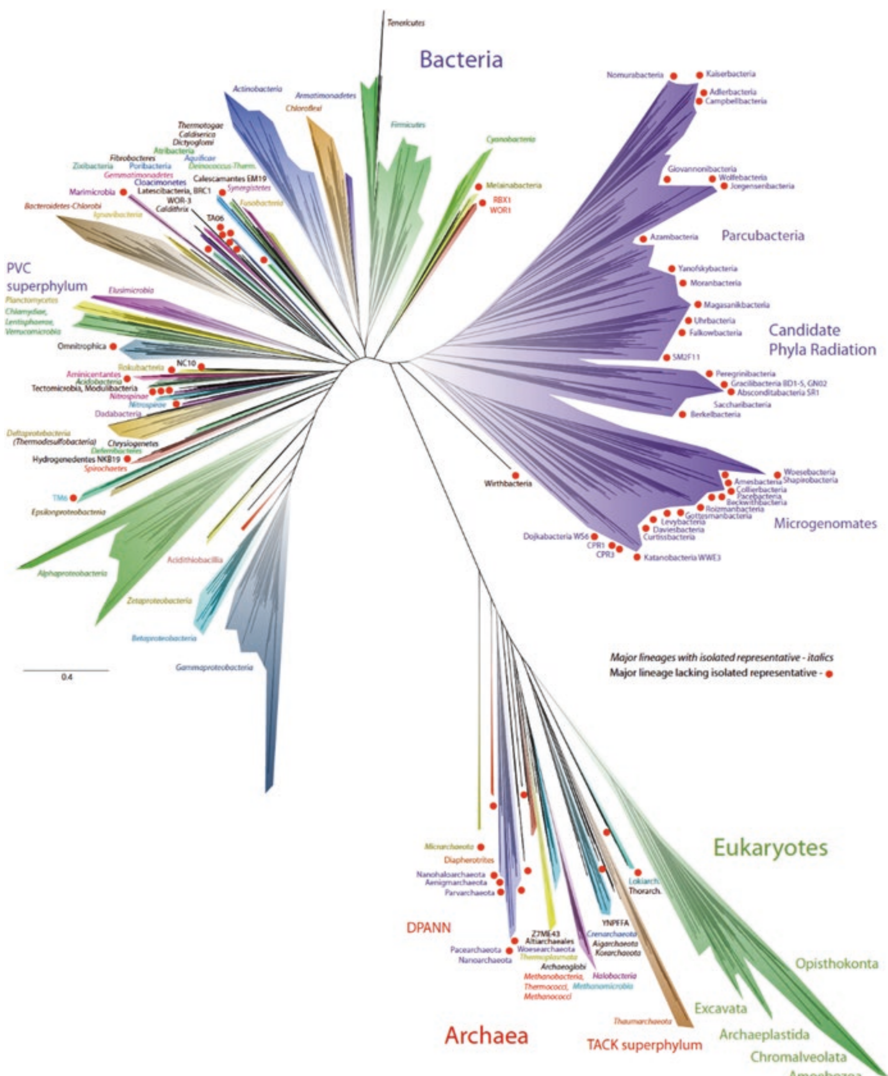


Fig. 18.3 Tree depicting the three domains of life (Source: Hug et al. 2016)

18.8.2 Industrial Application

Industries are now focusing on exploring the uncultured microbes, as prokaryotes are easy to screen by functional analysis using metagenomic approach. The literature published also reveals that bacterial lineage has the largest biodiversity. Thus, different industries are now motivated to exploit the large and diverse environment to isolate previously uncultivated microbes. Global scenario shows support towards the field of biotechnology in order to sustain the future of industries in this modern

era. That is why there is a need to identify novel enzymes and develop new process and products with their unique applications. So, taking these enzymes (biocatalyst) in consideration with respect to the industrial application, there are certain sets of parameters which are needed to be analysed. These parameters are categorised into four segments such as the following (Lorenz and Eck 2005):

18.8.2.1 Activity

Comprises of turnover frequency (k_{cat}), specific activity (kat/kg, U/mg), temperature profile and pH profile

18.8.2.2 Stability

Comprises of temperature stability, pH stability, ingredient/by-product stability and solvent stability

18.8.2.3 Efficiency

Comprises of space-time yield, product inhibition, ingredient/by-product inhibition and producibility/expression yield

18.8.2.4 Specificity

Comprises of substrate range, substrate specificity ($K_{m,}$ k_{cat}/K_m), substrate conversion (%), yield, substrate regioselectivity and enantioselectivity

The enzyme which satisfies the above parameters is regarded as the ideal biocatalyst. Firstly, the enzyme was extracted from yeast, fungi (filamentous), and very few numbers of culturable microbes (Burton et al. 2002). The novelty in the enzyme helps the industries to prevent the competition for applying for a patent and gain their intellectual property right. The novel starch liquefaction enzyme, α-amylase, which is stable at pH 4.5 and temperature of 95 °C with the length of 461 amino acids isolated from *Bacillus licheniformis* (mesophilic bacteria) has been patented by the number 5,958,672, which depicts the fact of novelty (Richardson et al. 2002). The diversity of the microbes is beneficial for both pharmaceutical and fine-chemical industries, as diversity enables them to set up the sets of multiple and diverse enzymes for biotransformation. The multiple and diverse sets of enzymes are needed to work in the strict timeline for evaluating the feasibility of biosynthetic catalyst against the traditional synthesised synthetic chemicals (Homann et al. 2004). Elusive metabolites are desired by many pharmacologists as the microbes are isolated from the complex consortium or niches which are complex for reconstruction in in vitro conditions (Piel 2004). Many reports have been published which state that these problems to cultivate microbes can be overcome either by creating the replica of natural habitat or by microencapsulating the single cell for communication among interspecies and cloning and studying the heterologous expression of gene (biosynthetic) which encodes for the desired secondary metabolite which emerges as the easy and reproducible method for assessment of potential of biosynthetic metabolites (Kaeberlein et al. 2002; Zengler et al. 2002). The major role of metagenomics in industrial biotechnology is discussed below.

18.8.3 Industrial Enzymes

Amylase, cellulase, lipase, protease, xylanase and other enzymes are of industrial importance. As the demand of these enzymes is increasing, metagenomic approach has emerged as one technology which has the ability to meet this industrial demand (Lorenz et al. 2002; Schloss and Handelsman 2003; Coughlan et al. 2015). Table 18.2 illustrates the metagenomic isolated enzyme from different environments.

18.8.4 Antibiotics and Bioactive Compounds Obtained

Turbomycins A and B were isolated by Gillespie and his colleagues from the metagenomic library, which proved the feasibility to use metagenomic approach to explore novel antimicrobial compounds (Gillespie et al. 2002). *Bor* gene cluster which isolated from the soil by Chang and Brandy was found to encode indolotryptoline compound, which belongs to the small and relatively rare family of the natural product which has a persuasive effect on the different cancerous cell line (Chang and Brady 2013). These discoveries have prompted the search operation to identify the novel drugs of medical importance, which were supported by various studies. The metagenomic approach is employed in association with homology- or functional-based method to explore bioactive compounds. Heterologous expression study is conducted by synthesising the novel molecule encoded by novel sequences obtained with the help of homology-based screening (Banik and Brady 2010). Thus, there is need to develop the expression system which should be highly selective, specific and sensitive and work on high-throughput programme so that we can completely explore the metagenomic libraries. METREX is such a designed system which comprises a host which carries GFP reporter gene that shows sensitivity to compound as resultant; there is quorum sensing. By following this approach, we can isolate the gene from the metagenomic library which encodes for compound which induces fluorescence when interacted with the reporter. Even though small fraction of compounds has been recognised till date by employing culture-independent approach, with addition to that, the preliminary studies signify that uncultured bacteria are the rich source of novel bioactive compounds (Bashir et al. 2014). Table 18.3 provides the information about the bioactive compounds and antibiotic identified by the metagenomic approach.

18.8.5 Personalised Medicine and Xenobiotic Degradation

The study of the mechanism of xenobiotics, especially the antibiotics which are vital on the microbiota of human gut, is important for understanding the drug resistance mechanism or the gene which is responsible for the risk of increasing drug resistance. Thus, there is a need for synthesising the drug which is effective and has minimum chance resistance by the infecting pathogens. By the enlightening, the mechanism of xenobiotic resistance and metabolism of the active microbiome of the

Table 18.2 Some of the industrial important enzymes identified by the metagenomic approach

Enzyme	Method				Library host	Country	Environment	References
	Activity-based screening	Function-based screening	Functional-based phagemid library/lambda phage library	PIGEX-based screening				
Lipolytic enzyme	√	–	–	–	*E. coli*	Germany	Top soil	Henne et al. (2000)
α-Amylase	–	√	–	–	*E. coli*	–	Sea water and acid soil	Richardson et al. (2002)
β-Agarase	√	–	–	–	*E. coli*	Germany	Top soil	Voget et al. (2003)
Lipolytic clones (esterase/lipase)	√	–		–	*E. coli*	Korea	Top soil	Lee et al. (2004)
Nitrilase genes	–	–	√	–	*E. coli*	–	Top soil and water	Robertson et al. (2004)
Esterases	√	–	–	–	*E. coli*	Mediterranean Sea	Seawater	Ferrer et al. (2005a)
Esterases, endo-β-1,4-glucanases and cyclodextrinase	–	–	√	–	*E. coli*	New Zealand	Cow rumen	Ferrer et al. (2005b)
ß-Glucanases	–	√		–	*E. coli*	New Zealand	Mouse bowel	Walter et al. (2005)
Esterase	√	–	–	–	*E. coli*	Indonesia	Mud sediment-rich water	Rhee et al. (2005)
Esterase	√	–	–	–	*E. coli*	Germany	Drinking water and top soil	Elend et al. (2006)

(continued)

Table 18.2 (continued)

Enzyme	Method				Library host	Country	Environment	References
Esterase	√	–	–	–	*E. coli*	Korea	Top soil	Kim et al. (2006)
Glycosyl hydrolase	–	–	√	–	*E. coli*	–	Cow rumen	Palackal et al. (2007)
Fibrinolyticmetalloprotease	√	–	–	–	*E. coli*	Korea	Mud	Lee et al. (2007)
Lipase	√	–	–	–	*E. coli*	Germany	Oil-contaminated top soil	Elend et al. (2007)
Esterase	√	–	–	–	*E. coli*	China	Seawater	Chu et al. (2008)
Cellulase	–	√	–	–	*E. coli*	China	Top soil	Jiang et al. (2009)
ß-Galactosidase	–	√	–	–	*E. coli*	China	Top soil	Wang et al. (2010)
Amidase	–	–	–	√	*E. coli*	Japan	Activated sludge	Uchiyama and Miyazaki (2010)
Tannase	√	–	–	–	*E. coli*	China	Top soil	Yao et al. (2011)
Proteases	√	–	–	–	*E. coli*	Mongolia and China	Sediments	Neveu et al. (2011)
Protease	√	–	–	–	*E. coli*	Tamil Nadu	Goat skin	Pushpam et al. (2011)
Esterase	√	–	–	–	*E. coli*	Korea	Compost	Kang et al. (2011)
Lipolytic enzyme	√	–	–	–	*E. coli*	Germany	Top soil	Nacke et al. (2011)

Lipase	√	–	–	–	*E. coli*	Brazil	Top soil	Faoro et al. (2012)
Esterase		√	–	–	*E. coli*	China	Cow rumen	Cheng et al. (2012)
Xylanase		√	–	–	*E. coli*	China	Cow rumen	Cheng et al. (2012)
ß-Galactosidase		√	–	–	*E. coli*	China	–	Wang et al. (2012)
Esterase	√	–	–	–	*E. coli*	China	Top soil and water	Ouyang et al. (2013)
Carboxylic ester hydrolases	√	–	–	–	*E. coli*	Belgium	Top soil	Biver and Vandenbol (2013)
Protease	√	–	–	–	*E. coli*	Belgium	Top soil	Biver et al. (2013)
Glycotransferase		√	–	–	*E. coli*	Germany	Elephant faeces and tidal flat sediment	Rabausch et al. (2013)
Lipase	√	–	–	–	*E. coli*	China	Marine sediment	Peng et al. (2014)
ß-Galactosidase	–	√	–	–	*E. coli*	Denmark	Ikaite columns of submarine SW Greenland	Vester et al. (2014)
α-Amylase	–	–	–	√	*E. coli*	India	Cow dung	Pooja et al. (2015)
Esterase	–	√	–	–	*E. coli*	Portugal	Water, sediment and biofilms	Leis et al. (2015)
β-*N*-acetylhexosaminidases	–	√	–	–	*E. coli*	Denmark	Human milk oligosaccharides	Nyffenegger et al. (2015)
Esterase	–	√	–	–	*E. coli*	China	Marine sediment	Hu et al. (2015)

(continued)

Table 18.2 (continued)

Enzyme	Method				Library host	Country	Environment	References
Esterase	–	√	–	–	*E. coli*	Yunnan	Surface of chestnut grove	Gu et al. (2015)
Rhodanese	–	√	–	–	*E. coli*	India	Top soil	Bhat et al. (2015)
Iota-carraageenase and esterase	–	√	–	–	*E. coli*	Belgium	Top soil	Martin et al. (2016)
Proteases	–	√	–	–	*E. coli*	Mexico	Underground water	Apolinar–Hernández et al. (2016)
Cellulases	–	√	–	–	*E. coli*	Hong Kong	Anaerobic digestion sludge	Yang et al. (2016)
Esterase	–	√	–	–	*E. coli*	Siberia	Permafrost sample from bore hole	Petrovskaya et al. (2016)
Chitin deacetylase	–	√	–	–	*E. coli*	Arctic Ocean	Deep-sea sediment	Liu et al. (2016)
Polyhydroxyalkanoate synthase	–	√	–	–	*E. coli*	Perak	Top soil	Tai et al. (2016)
Esterase	–	√	–	–	*E. coli*	Russia	Hot spring mud	Zarafeta et al. (2016)
Carboxylesterases	√	–	–	–	*E. coli*	–	Marine environments, soils and waste treatment facilities	Popovic et al. (2017)
β-Galactosidases	–	√	–	–	*E. coli*	Canada	Top soil	Cheng et al. (2017)

Table 18.3 Some of the bioactive compounds and antibiotic identified by the metagenomic approach

Bioactive	*Method*	*Host*	*Country*	*Environment*	*References*
Biotin	SBS	*E. coli*	Germany	Horse excreta	Entcheva et al. (2001)
Pederin	TSBS	–	Germany	Paederus beetles	Piel (2002)
Vibrioferrin	FBS	*E. coli*	Japan	Tidal sediment	Fujita et al. (2011)
Borregomycins A and B	HGS	–	USA	Top soil	Chang and Brady (2013)
Antibiotic	*Method*	*Host*	*Country*	*Environment*	*References*
Terragine	ABS	*Streptomycetes*	Canada	Top soil	Wang et al. (2000)
Turbomycins A and B	ABS	*E. coli*	USA	Top soil	Gillespie et al. (2002)
Indirubin	FBS	*E. coli*	Korea	Top soil	Lim et al. (2005)
Beta-lactamases	FBS	*E. coli*	Alaska	Top soil	Allen et al. (2009)
Fasamycins A and B	ABS	*E. coli*	USA	Top soil	Feng et al. (2012)

SBS, selection-based screening; *TSBS*, targeted sequencing-based strategy; *FBS*, function-based screening; *HGS*, homology-guided screening; *ABS*, activity-based screening

human gut not only help us to understand the host-microbe interaction and biochemistry among them but additionally also provide the hints for understanding variation in the patient response to drug efficacy as well as toxicity. This matter is dealt with the metagenomics which allows the analysis of the cumulative genome of the bacterial community, especially the microbiome of the gut (Rankin et al. 2016; Spanogiannopoulos et al. 2016). Maurice et al. enlighten the relation of the gene expression and metabolism of the unique active microbes of the gut which promptly gets altered by antibiotics and host-targeted drugs (Maurice et al. 2013). These verdicts bring to light the unpremeditated effects of xenobiotics and signified the role of microbiota which should be considered as a factor during the development of the personalised medicines. The assimilated characteristics of microbiome of gut against xenobiotics which can eventually be employed for designing the new diagnostic assay which can predict the pharmacokinetics of the drug and therapeutic intrusions (Bashir et al. 2014).

18.8.6 Bioremediation Facilitated by Biosurfactant

For the treatment of petroleum hydrocarbons which are present in the oil spills, presently chemical surfactants are used for emulsification, which increases their solubility and aids in consequent deprivation through oil-degrading bacteria. On the

other hand, the chemical surfactants which have been used for bioremediation purpose are excluded for being toxic and less biodegradable (Kennedy et al. 2011). Hence, the biosurfactants have emerged as an environment-friendly substitute which is not toxic as compared to the chemical surfactants (Pacwa-Płociniczak et al. 2011). Generally, biosurfactants are stated to be the molecule which has amphipathic nature, i.e. it has the hydrophobic as well as hydrophilic group that separates favourably at the interface of two fluids which have varied degree of polarity and hydrogen bonding, for example, water and oil or water and air peripheries (Joshi and Desai 2010). Metagenomics enables us to screen the clones that have ability to synthesise the biosurfactants from the DNA library constructed from sample contaminated with petroleum (water, soil, etc.). Different screening assays have been designed for the screening of metagenomic clone which have ability to produce biosurfactant, such as atomised oil assay, in which fine drop of oil is put on the surface agar plate and is monitored immediately for the biosurfactant production as halos near metagenomic clone (Burch et al. 2010). Another such approach is oil-coated agar plate in which biosurfactant-producing clones are recognised by emergence of emulsified halo (Kennedy et al. 2011); haemolytic activity is also assessed as it is also an indication for biosurfactant-producing clone as in this approach haemolytic cell lysis is observed (Varjani et al. 2014). In blue-agar method in which agar comprises of mineral salt with 2% carbon source, 0.0005% CTAB and 0.002% methylene blue, dark blue halo is the indication of biosurfactant production (Bashir et al. 2014). Function-based approach, SIGEX, is a novel method that facilitates in the screening of metagenomic libraries (Bashir et al. 2014). Hence, by using the above discussed screening approaches, it is expected that we will able to identify the cluster of novel genes which produce biosurfactant, and it will also speed up the improvement of bioremediation methods which use biosurfactants. Moreover, metagenomic-based bioremediation research is taking place. Recently, the report of alkane hydroxylase enzyme identified by metagenomic approach is used for degrading hydrocarbons (Paul et al. 2005).

18.9 Conclusion

Culture-dependent screening methods limit the chance to get the newer future molecules with more stability and functionality. Developments of tools and techniques to screen direct DNA-based screening will improve the chances to get the future novel and more stable molecules. Metagenomics along with the molecular biology and microbiology will open new dimensions for future research and developments for achieving the sustainability.

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19 Bacteriophage-Mediated Biosensors for Detection of Foodborne Pathogens

Vipin Singh

Abstract

Food is the primary source of energy for living organisms. However, depending on a variety of factors, including source, freshness, and storage conditions, food may undergo spoilage by microorganisms and cause foodborne disease outbreaks that can be detrimental to community and human health. There is thus a need for developing rapid, accurate, and reliable methods for the detection of foodborne pathogens. Bacteriophages (phages), viruses that infect and replicate in bacterial cells, can be exploited as bio-receptors in biosensor detection systems, serving as a promising prospect in biotechnology. Phage-mediated detection methods are reliable, quick, precise, sensitive, selective, and cost effective. Bacteriophage-based biosensors are being used to sense pathogens at significantly low bacterial cell concentrations, as well as being used for monitoring the health and safety aspects of food in real time. In this chapter, we review recent progress in phage-based sensing strategies for developing biosensor technology.

Keywords

Biosensor · Bio-receptor · Foodborne pathogen · Bacteriophage

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19.1 Foodborne Illnesses

The paramount concern of the food industry is to ensure the supply of nutritious and hygienic food commodities to consumers. The food industry is particularly wary about the presence of pathogenic microorganisms, like bacteria, with which food can become naturally or accidentally contaminated. The ingestion of pathogen-contaminated foodstuffs that cause disease outbreaks arising from toxins has been documented in many countries (WHO 2005, 2007a, b; Velusamy et al. 2010). Thus, ensuring hygenic food security is of immense significance for the welfare of a country's population. Food recalls are not uncommon when a food commodity is of dubious quality owing to contamination with pesticides or contamination with pathogens, e.g., *Escherichia coli* O157:H7, which causes many diseases in humans. In the United States, contaminated food causes many illnesses annually; illness arising from the ingestion of *E. coli* O157:H7-contaminated food was first reported in 1983, and this microbe is now a major cause of foodborne problems in developed countries (Riley et al. 1983; Wells et al. 1983; WHO 2005, 2007a, b; Velusamy et al. 2010). The infection dose of pathogens is very low (~10 bacteria) and these pathogenic bacterial strains can become drug-resistant. Therefore, priority needs to be accorded to monitoring the health and safety aspects of food, and generating new, quick, and timely detection methods for pathogens in food and water (Singh et al. 2013).

Some common foodborne pathogens, along with some reported incidents of disease outbreaks caused by pathogenic microorganisms, are listed in Table 19.1. To ensure food safety, good manufacturing practices, critical control points, and food codes that reduce contamination in food, and also prevent and identify pathogenic

Table 19.1 Recent incidences of foodborne disease outbreaks caused by pathogenic microorganisms (Velusamy et al. 2010)

Pathogens	Place and year	Source	Diseases
Escherichia coli	Norway (2006), USA (2007), and Europe (2011)	Hamburger patties, chicken, and milk	Hemorrhagic colitis, stomach pain, diarrhea, nausea, fever, and headache
Salmonella spp. and *Shigella*	Norway (2004), Japan (2004), USA (2004), Thailand (2005), Germany (2006), and South Korea (2007)	Egg, squash, seafood, cake, rice, chicken, ice cream, milk, and dairy products	Headache, fever, nausea, and abdominal pain
Listeria monocytogenes	USA (2002), Canada (2004), and Japan (2001)	Cheese, unpasteurized milk, contaminated vegetables, soft cheese, improperly processed ice cream	Listeriosis, fever, intense headache, nausea, and vomiting
Campylobacter jejuni	USA (2004) and Japan (2005)	Chicken, raw milk, seafood, poultry	Campylobacteriosis, fever, headache, muscle pain, abdominal pain, diarrhea, and nausea

microorganisms that contaminate food and water, are necessary (Piatek and Ramaen 2001; Umali-Deininger and Sur 2007; Jin et al. 2008; Mucchetti et al. 2008).

19.2 Major Pathogens

The World Health Organization (WHO) has defined foodborne illnesses as those that cause either infections or are poisonous in nature and those that are introduced by contaminated compounds that enter the body by the ingestion of food and water. The major foodborne pathogens that have been identified as causes of foodborne diseases are *Escherichia coli*, *Listeria monocytogenes*, *Salmonella*, *Yersina enterocolitica, Clostridium botulinum*, and *Campylobacter* (Velusamy et al. 2010). Some other pathogenic microorganisms that cause foodborne diseases are *Clostridium perfrigens*, *Shigella*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* (Alocilja and Radke 2003; Chemburu et al. 2005; Kay et al. 2008; Velusamy et al. 2010). The characteristic attributes of some of these pathogens are enumerated in the following section.

19.2.1 Escherichia coli

The *E. coli* cell is about 2 μm long and 0.5 μm in diameter, with a cell volume of 0.6–0.7 $μm^3$ (Escherich 1885; Kubitschek 1990). *E. coli,* a gram-negative, facultative anaerobe, is a coliform bacterium of the genus *Escherichia* and is widely distributed in the intestines of humans, birds, and animals, where it is found in the lower intestinal flora and maintains the physiology of the healthy host (Ewing 1986).

E. coli is a member of the family *Enterobacteriaceae* (Neill et al. 1994). While most strains of *E. coli* are harmless, opportunistic pathogens and pathogenic strains are also prevalent, and when ingested, these cause urinary tract infections, gastrointestinal disorders, and neonatal meningitis in humans. Pathogenic strains of *E. coli* have also been implicated in hemolytic uremic syndrome (HUS), septicemia, pneumonia, mastitis, and peritonitis. A procedure called serotyping is commonly adopted for the subdivision of *E. coli* types. *E. coli* strains bear unique serological traits and hence are serotyped according to their surface antigens, as: O (heat-stable somatic antigens), K (heat-stable capsular antigens), and H (heat-labile flagellar antigens). Currently, approximately 167 serological O antigens, 74 serotypes, and 53 H antigens have been identified (Lior 1994).

Enteric *E. coli* has serological and virulence properties. Enteric *E. coli* infections are divided into six pathotypes based on the capacity of the *E. coli* to produce toxins, virulence factors, clinical disease, and pathogenicity profiles. The six pathotypes are enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic (EHEC), enteroaggregative, and diffuse-adhering *E. coli*. The mechanisms of the pathotype and the symptoms produced by these groups are distinct and show some overlapping characteristics. Enterohemorrhagic *E. coli* of serogroup O157: H7 is a

human pathogen that causes bloody diarrhea and HUS (Donnenberg and Whittam 2001; Ray and Bhunia 2007). Fecal-oral transmission of pathogenic bacteria causes disease, since these bacteria are normally present at very high levels (10^6/gram of sample) in the large intestine, and for long periods. *E. coli* has been used as an index organism for determining possible contamination and presence of pathogens in water and food. The presence of these bacteria in food or water indicates possible contamination of either animal or human fecal origin.

19.2.2 Salmonella

Salmonella is a genus of rod-shaped Gram-negative bacteria, belonging to the phylum *Proteobacteria* and the family *Enterobacteriaceae*. The genus consists of two species, *S. bongori* and *S. enterica*; *S. enterica* is divided into six subspecies: *arizonae, diarizonae, enterica, salamae, houtenae,* and *indica. Salmonella enterica* causes food poisoning by contaminating poultry products (Uzzau et al. 2000; Rabsch et al. 2001). The two most common serovars responsible for infection are *S. typhimurium* and *S. enteritidis*, which are isolated from poultry (Baumler et al. 2000; Guard-Petter 2001; Poppe 2000).

Because of the overuse of antibiotics, *Salmonella* isolates are resistant to multiple antibiotics. This resistance is a major concern and infection with such isolates is a huge problem in developing countries (Boyle et al. 2007). *Salmonella* contamination occurs via the fecal-oral transmission mode and causes such conditions as diarrhea, nausea, abdominal pain, fever, and vomiting in humans and animals (Giannella et al. 1972, 1973; Blaser and Newman 1982). The number of infectious bacteria required to cause disease shows great variation—from 30 to 10^9 (Morgan et al. 1994; Vought and Tatini 1998; Mead et al. 1999; Majowicz et al. 2010).

19.2.3 Listeria

Listeria monocytogenes is a Gram-positive bacterium of the family *Listeriaceae* and is encountered in soil, water, and rotting plant materials. *L. monocytogenes* is capable of growing at 0 °C, multiplying at refrigeration temperatures, and surviving in damp areas; it can stay alive even on glass materials and stainless steel (Al-Zoreky and Sandine 1990; Genigeorgis et al. 1991). Most common illnesses caused by *L. monocytogenes* are associated with refrigerated foods that are not recooked before consumption. *L. monocytogenes* causes listeriosis, which ranks third in the total number of deaths caused by pathogenic bacteria and third in death rates caused by foodborne infection, ranking above other pathogenic bacteria such as *Salmonella* and *Clostridium botulinum* (Ramaswamy et al. 2007). The symptoms and signs of listeriosis range from a mild flu-like illness, muscle aches, nausea, diarrhea, and fever to nervous system involvement with loss of balance, headache, and confusion (Farber and Peterkin 1991; Gray et al. 2004).

L. monocytogenes infection also causes meningitis in pregnant women, children, and the elderly.

19.2.4 Campylobacter

Campylobacter jejuni infection causes disease in humans and animals and is recognized as the main cause of foodborne diseases. *Campylobacter*, which is commonly found in animal feces, is a gram-negative bacterium whose infections (campylobacteriosis) trigger the development of Guillain-Barré syndrome and reactive arthritis. Consumption of improperly cooked and undercooked meat and poultry causes campylobacteriosis (Kist 1985). In 1886, Theodore Escherich detected microorganisms similar to *Campylobacter* in the stool samples of children with diarrhea (Vandamme et al. 2010). In 1996, 46% of surveyed laboratories confirmed that their reported cases of bacterial gastroenteritis were caused by *Campylobacter*. According to the WHO surveillance network program, campylobacteriosis (at 46%) was followed in incidence by salmonellosis (28%), shigellosis (17%), and *E. coli* O157: H7 infection (5%) (Altedruse et al. 1999). Stern and Line (1992) reported that 98% of retail chicken meat samples were contaminated with *C. jejuni*. Rohrbach et al. (1992) found that 12% of raw milk samples from dairy farms were contaminated with *C. jejuni,* while Hudson et al. (1984) reported that raw milk is presumed to be contaminated by bovine feces; however, direct contamination of milk as a consequence of mastitis also occurs.

19.3 Techniques for Determination of Contamination by Pathogens

19.3.1 Conventional Methods

For the detection of pathogenic bacteria, standard conventional microbiological methods—such as bacterial colony counting, biochemical and immunological methods, and the polymerase chain reaction (PCR) molecular biological method—are employed, but these methods are time consuming. Thus, there is a need for developing quick and sensitive sensing systems. In this context biosensing platforms, which detect pathogens at different concentrations, and are inexpensive, may be considered (Chemburu et al. 2005; Alocilja and Radke 2003; Pettya et al. 2006; Naidoo et al. 2012).

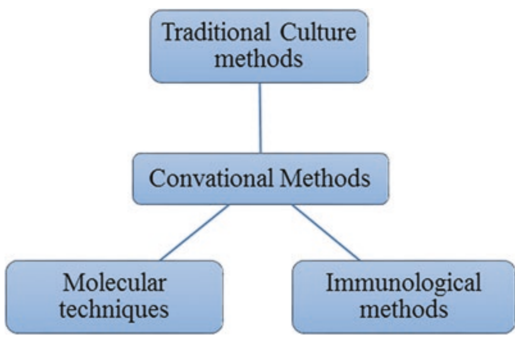

Fig. 19.1 Conventional methods for the detection of foodborne pathogens (Velusamy et al. 2010), with permission from

Numerous conventional and newer methods are used currently to detect food-borne pathogens (Fig. 19.1).

19.3.2 Traditional Culture Methods

With traditional culture methods, pathogens present in a sample are cultured on different types of media in order to establish their presence and identify them. The media may be selective or differential for the growth of specific bacteria, or they may be media that show different phenotypic characteristics (DeBoer and Beumer 1999; Artault et al. 2001).

There are two main culture strategies— quantitative and qualitative. In quantitative culture, individual microorganisms will grow to form specific colonies that can be counted to evaluate the number of microorganisms. With the qualitative culture method, the target colonies of microorganisms that grow on selective/differential media are called "presumptive" colonies. *L. monocytogenes* organisms are detected by using culture methods. Pathogens that are detected by culture methods are *Staphylococcus*, *Salmonella*, *L. monocytogenes*, *E. coli*, and *Campylobacter* (Ayçiçek et al. 2004; Sanders et al. 2007).

19.3.3 Immunological Methods

Immunological methods are those that depend upon the interaction of an antigen (protein or the entire microorganism) with an antibody that is specific to the particular antigen. Pathogens detected by immunology-based methods are *E. coli*, *Salmonella*, *L. monocytogenes*, *Staphylococcal enterotoxins*, and *Campylobacter* (Rasooly and Rasooly 1998; Abdel-Hamid et al. 1999a,b; Gangar et al. 2000; Siragusa et al. 2001; Chen and Durst 2006; Che et al. 2001; Borck et al. 2002; Aldus et al. 2003; Valdivieso-Garcia et al. 2003; Bennett 2005; Schneid et al. 2006; Churchill et al. 2006; Hibi et al. 2006; Jechorek and Johnson 2008; Hochel et al. 2007). Further immunological methods, such as enzyme immunoassays and enzyme-linked immunosorbent assays, have been developed for the detection of pathogenic microorganisms (Mattingly et al. 1988; Beumer and Brinkman 1989; Borck et al. 2002; Palumbo et al. 2003; Bennett 2005). Other immunological techniques that can be utlilized include flow injection immunoassays, bioluminescent immunoassays, immunomagnetic chemiluminescence and separation, immunochromatography tests, immunoprecipitation and agglutination, radioimmunoassays, and western blotting (Abdel-Hamid et al. 1999b; Valdivieso-Garcia et al. 2003; Gehring et al. 2006; Shim et al. 2007; Dickson and Chen 2001; Hudson et al. 2001; Refseth et al. 2001; Feldsine et al. 1997; Matar et al. 1997; Rasooly and Rasooly 1998).

19.3.4 Molecular Techniques

Molecular techniques are those that involve the use of DNA strands or probes for the identification or detection of pathogenic organisms. PCR-based methods, first described in the 1980s, are now often used for bacterial detection (Mullis et al. 1986; Lazcka et al. 2007). These methods are popular for their exquisite sensitivity and speed. Several versions of PCR have been developed, including real-time PCR, multiplex PCR, and reverse transcriptase-PCR (Deisingh and Thompson 2004; Rodriguez-Lazaro et al. 2005; Jofre et al. 2005).

Some of the commercially available kits for *E. coli* detection are listed in Table 19.2. One method used is the random amplified polymorphic DNA technique, which utilizes PCR amplification with arbitrary sequence primers to produce arrays of anonymous DNA fragments of a specific organism (Choi and Lee 2004; Perry et al. 2007; Messelhausser et al. 2007). PCR methods are used to detect pathogens such as *E. coli* O157: H7, *S. aureus*, *Listeria*, *Salmonella*, *Bacillus cereus*, *Yersinia enterocolitica*, and *C. jejuni* (Yaron and Matthews 2002; Kim et al. 2007; Malorny et al. 2007; Ronner and Lindmark 2007; Perry et al. 2007; Chen and Knabel 2007; Murphy et al. 2007; Riyaz-Ul-Hassan et al. 2008).

19.4 Biosensors for Detection of Foodborne Pathogens

Biosensors are analytical devices that consist of a biological recognition element (also called a bio-receptor), and a transducer that converts the biological signal into a readable signal that is then displayed and analyzed (Fig. 19.2). The types of bio-receptors that are generally used include phage, whole microbial cell, enzyme,

Table 19.2 Commercially available *Escherichia coli* detection kits (Velusamy et al. 2010)

Detection methods	Company and country	Time period	References
Culture method	Vermicon Identification Technology, Germany	Within 3 h	
ALOA® count method for *Listeria*	Biomerieux, France	Up to 3 days	Artault et al. (2001)
VIDAS® *E. coli* (ECO) (phage immunoassay)	Biomerieux, India	6–24 h	biomerieux-industry.com
Coli plate (culture method)	Biotecon Diagnostics, Potsdam, Germany	3–4 h	Lifshitz and Joshi (1998)
E. coli detection kit (Molbio-PCR)	Himedia, France	4–5 h	Himedia.com
PCR-based method	Norgenbiotek, Canada	3–4 h	Norgenbiotek.com
Atlas *E. coli* (PCR-based method)	Roka Bioscience, USA	4–5 h	Rokabio.com
MicroSEQ *E. coli* detection kits (PCR-based method)	Thermo Fisher Scientific, USA	8–10 h	Wong et al. (2012)
3 M™ Tecra™ *E. coli* (immunoassays)	3 M, USA	20–26 h	Montgomery and David (2014)

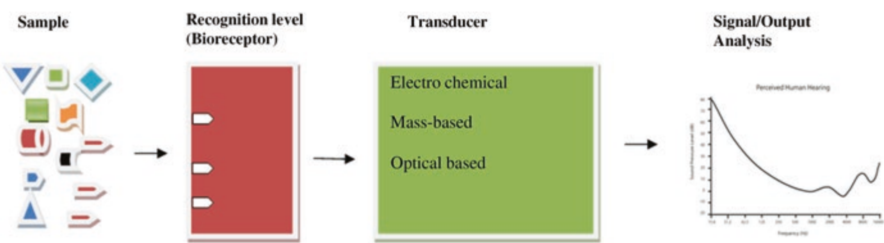

Fig. 19.2 Essential components of biosensors (Velusamy et al. 2010)

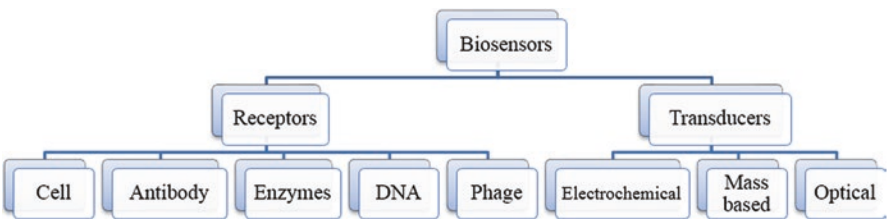

Fig. 19.3 Classification of biosensors (Velusamy et al. 2010)

antibody, and nucleic acid types. Transducers are either electrochemical, mass-based, or optical, or combinations of these types (McNaught and Wilkinson 1997; Velusamy et al. 2010). In the first biosensor, devised by Professor Leland C. Clark Jnr. in 1962, glucose oxidase was entrapped at an oxygen electrode (subsequently termed the Clark electrode), using a dialysis membrane (Clark and Lyons 1962).

19.4.1 Classification of Biosensors

Biosensors are classified according to the type of bio-receptor and transducer used. Classification by bio-receptor depends upon the entity constituting the recognition element, such as an enzyme, antibody-antigen, bacteriophage, tissue, DNA, or whole cell. Types of transducers used with different biosensors depend on the the transducer signals. i.e., electrical, thermal, or optical (Fig. 19.3) (McNaught and Wilkinson 1997).

19.4.2 Bio-receptors/Biological Recognition Elements

Bio-receptors or biological recognition elements play a crucial role in the specificity and selectivity of biosensor technologies. Diverse microbial entities have been utilized as bio-receptors and these are briefly enumerated in this section.

19.4.2.1 Antibodies

Antibodies are the most common biological recognition elements used in biosensors. Antibodies are immobilized on a working electrode surface (Lazcka et al. 2007), which then facilitates antigen and antibody interaction (VoDinh and Cullum 2000). Antibody-based techniques are of two types, the first being a direct single-step method, in which a fluorescent tag-labeled antibody reacts directly with the antigen (Coons et al. 1942). The second type is an indirect method, in which the first layer of unlabeled primary antibody reacts with the antigen, followed by a second layer of labeled secondary antibody that reacts with the primary antibody. The second-layer antibody is labeled with fluorescent dye or an enzyme (Weller and Coons 1954). A surface plasmon resonance (SPR) method is also used to detected foodborne pathogens, with the employment of antibody bio-receptors, magneto-elastic (ME) resonance sensors, and immune sensors (Taylor et al. 2006; Waswa et al. 2007; Guntupalli et al. 2007; Tokarskyy and Marshall 2008).

19.4.2.2 Enzymes

Enzymes employed as biological recognition elements in biosensors are affixed to the working electrode. Enzymes are highly specific and selective in their catalytic activity and in binding with a suitable substrate. Enzymes used as bio-receptors thus provide high specificity, and their catalytic action helps to quantitatively determine pathogenic bacteria (Vo-Dinh and Cullum 2000). Enzymes have also been used as indirect bio-receptors to label the primary antibody for the detection of pathogenic bacteria such as *L. monocytogenes*, *E. coli*, and *C. jejuni* (Chemburu et al. 2005).

19.4.2.3 Bacteriophages

Bacteriophages can be exploited as bio-receptors (Fig. 19.3) in biosensors, because they are natural predators of bacteria. The phage virus particles are amplified inside bacteria, producing a number of copies that accentuate the infection process. Phages have a unique property in that they are very selective in lysing and killing their specific hosts. Phages have been used as biological recognition elements, as they can infect bacterial pathogens such as *E. coli, Staphylococcus*, *Campylobacter*, and *Bacillus* (Balasubramanian et al. 2005; Balasubramanian et al. 2007; Huang et al. 2008; Singh et al. 2009).

19.4.2.4 Nucleic Acids

Nucleic acid recognition bio-receptors, especially deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), serve as biochips. Nucleic acid-based DNA and RNA biosensors are simple, show rapid action, and are inexpensive. Nucleic acid-dependent biosensors have been successfully employed for the detection of bacterial pathogens such as *E. coli*, *Salmonella*, *Bacillus*, and *Campylobacter* (Uyttendaele et al. 1997; Lermo et al. 2007; Chen et al. 2008). DNA microarray techniques are also used in biochips for the detection of *Listeria*, *Campylobacter*, *S. aureus*, and *Clostridium* (Sergeev et al. 2004). A new advance in nucleic acid recognition is the utilization of peptide nucleic acid (Briones et al. 2004; Fan et al. 2007; Mateo-Marti et al. 2007)

19.4.2.5 Cellular Bio-receptors

Cellular bio-receptors consist of whole-cell, mitochondrial, or other cellular components, such as enzymes and proteins (Velusamy et al. 2010; Pancrazio et al. 1999). Mammalian cell bio-receptors are creating increasing interest for the detection of pathogens (Bhunia et al. 2007). Banerjee et al. (2008) reported a whole-cell sensing system with a collagen-encapsulated B-lymphocyte cell line as a biosensor for the rapid detection of pathogenic bacteria. Mitochondria are used as biosensors for calcium microdomains (Rizzuto et al. 1999). An optical whole-cell biosensor provided with *Chlorella vulgaris* has been designed for monitoring herbicides (Védrine et al. 2003). Whole-cell amperometric microbial biosensors have been developed for the detection of *Pseudomonas* sp. (Dubey and Upadhyay 2001; Campas et al. 2008).

19.4.3 Transducers

Transducers play an important role in the performance of biosensors as they convert biological signals to recordable signals for the analysis of data (Fig. 19.3). Different types of transducers are described below.

19.4.3.1 Optical Biosensors

Optical-based biosensors are fairly selective and sensitive for detection purposes. Optical detection methods may be based on factors such as light absorption, refraction, dispersion, and reflection; infrared light; Raman spectroscopy; SPR; chemiluminescence; fluorescence; and phosphorescence (Ko and Grant 2006). The application of optical-based techniques has been described for the detection of *Listeria*, *Salmonella*, *E. coli*, and *Clostridium botulinum* toxins (Ogert et al. 1992; Strachan and Gray 1995; DeMarco and Lim 2002; Ye et al. 2002; Simpson and Lim 2005; Ko and Grant 2006). DeMarco and Lim (2002) demonstrated a fiber-optic biosensor for the detection of *E. coli* O157: H7 in beef samples.

19.4.3.2 Raman and Fourier Transform Infrared Spectroscopy

Spectroscopy, which is based on a light-scattering technique, is used for the detection of pathogens, and spectroscopy at 785 nm is used to determine the presence of Gram-positive and -negative bacteria (Schmilovitch et al. 2005). Yu et al. (2004) developed a Fourier transform infrared spectrometry-based approach for bacterial identification and quantification of *Salmonella*, *Enterobacter*, *Citrobacter*, *Yersinia*, *Staphylococcus, E. coli*, *Listeria*, and *Klebsiella* (Schmilovitch et al. 2005; Davis et al. 2010a, b).

19.4.3.3 Surface Plasmon Resonance (SPR)

Surface plasmon resonance is a method in which plane polarized light is used for the irradiation of a sample surface (such as a metal film) creating reflections. SPR is used to measure changes in the refractive index that arise owing to biomolecular interactions on the transducer surface; changes in the resonance angle and wavelength are also measured. Biosensors based on SPR have been shown to detect the following pathogens: *Listeria*, *Salmonella*, *E. coli*, and *C. jejuni* (Koubova et al.

2001; Oh et al. 2003, 2004; Taylor et al. 2005, 2006; Meeusen et al. 2005; Subramanian et al. 2006; Waswa et al. 2007). Commercially available biosensors based on the SPR method for the identification of pathogens are SPREETA (Texas Instruments, USA) and BIACORE3000 (GE healthcare, Sweden), which have been used for the detection of *E. coli, L. monocytogenes,* and *Salmonella* (Bokken et al. 2003; Leonard et al. 2004).

19.4.3.4 Electrochemical Biosensors

Electrochemical biosensors, which are highly sensitive miniaturized detectors, can operate well in turbid media and have been used for the analysis and detection of pathogens. These biosensors can be subdivided into potentiometric, amperometric, galvanometric, impedimetric, conductometric, and cyclic voltammetry types. These biosensor types perceive changes in such observed parameters as current, voltage, impedance, conductance, and voltammetry, respectively.

19.4.3.5 Amperometric Methods

The amperometric method of detection is based on the production of a current when a potential develops between two electrodes; for example in a microfluidic sensor (Reymond et al. 2007). Detection of foodborne pathogens such as *E. coli*, *Salmonella*, *Listeria*, and *C. jejuni* (Brooks et al. 1992; Che et al. 1999; Crowley et al. 1999; Abdel-Hamid et al. 1999a,b; Chemburu et al. 2005; Yang et al. 2001; Chemburu et al. 2005) has been demonstrated with this technique.

19.4.3.6 Potentiometric Methods

Potentiometric biosensors utilize ion-selective electrodes. This method works on the detection of ion concentrations in the relevant sample solution. A potentiometric system contains three electrodes; one reference electrode, one counter electrode, and a working electrode in contact with the sample. Pathogen detection is based on changes in pH or in ion concentrations (Mackay et al. 1991; Gehring et al. 1998). Ercole et al. (2003) was used the potentiometric alternation biosensing (PAB) system based on light-addressable potentiometric system for the detection of *E. coli* cells.

19.4.3.7 Conductometric Methods

Conductometric methods monitor changes in electrical conductivity occurring in a solution. Muhammad-Tahir and Alocilja (2003a, b) reported the detection of *E. coli* and *Salmonella* spp. by a conductometric method. Pal et al. (2008) developed conductometric biosensors that involved a direct charge transfer method for the detection of *B. cereus* in food samples.

19.4.3.8 Impedimetric Methods

Impedimetric techniques are used for ascertaining a range of pathogenic bacteria. The impedance detection method measures changes in electrical current and charge transfer resistance over an electrode surface. This method is simple, less reagent-dependent, cost effective, sensitive, and specific for evaluating foodborne pathogens (Tully et al. 2008; Mejri et al. 2010, 2011; Rohrbach et al. 2012; Park et al.

2013). Yang et al. (2004) reported the detection of *Salmonella* by using impedance sensors. Impedance was recorded against bacterial growth time at four frequencies: 10 Hz, 100 Hz, 1 kHz, and 10 kHz, and impedance analysis showed a limit of detection between 10^5–10^6 colony-forming units (cfu)/ml. Shabani et al. (2008) reported the detection of *E. coli* bacteria by an impedance method for a T4 phage immobilized on an electrode as a bio-receptor; the limit of detection was approximately 10^4 cfu/ml.

19.4.3.9 Mass-Sensitive Method

The mass-sensitive method, in which transducer function is predicated on extremely small transmutations in mass, is very selective. In principle the method depends on the utilization of crystals, which can measure vibration at a categorical frequency oscillation; the method also depends on the electrical frequency and the mass of the crystal. The two main types of mass-predicated sensors are the quartz crystal microbalance (QCM) and the surface acoustic wave (Velusamy et al. 2010).

Su and Li (2005) confirmed the use of QCM sensors for the detection of *S. typhimurium* in chicken meat samples. The QCM sensing method can detect foodborne bacteria such as *L. monocytogenes*, while *E. coli* O157: H7 was detected using a surface acoustic wave method (Vaughan et al. 2001; Berkenpas et al. 2006).

Table 19.3 List of commercial biosensor transducers (Arora et al. 2011)

Transducer	Biological recognition element	Type of biosensor or measured parameter	Company name and location
Electrochemical		Potentiometric, conductometric, amperometric voltametric impedimetric	Malthus 2000 Analyzer (Malthus, Stoke-on-Trent, UK), Midas pro (Biosensori, Milan, Italy), Bactometer (Bactomatic, Princeton, NJ, USA)
Electrical	Enzymes, proteins, amino acids, nucleic acids: DNA, RNA, PNA, antibodies, antigens, specific genes, organelles, microbial cells, plant and animal tissues	Surface plasmon resonance, surface conductivity, electrolyte conductivity	Biosensing Instruments-SPRM200 (USA), IBIS Technologies (Netherlands), BioRed (USA), ICX Technologies (USA), GWC Technologies (USA), Sensata Technologies(USA), GE healthcare (Sweden)
Optical		Ultraviolet absorption, fluorescence emission, optical quantitative imaging adsorption, bioluminescence, chemiluminescence	Nanolane (France), Lumac Biocounter (Lumac B.V., Schaesberg, Netherlands), OWLS sensor (MicroVacuum Ltd, Hungary)
Mass-sensitive		Resonance frequency of piezocrystals, piezoelectric, surface acoustic wave	Unilite (Biotrace, Bridgend, UK), Axela (Canada)

DNA deoxyribonucleic acid, *PNA* peptide nucleic acid, *RNA* ribonucleic acid

Nanduri et al. (2007) developed a biosensor system to detect *E. coli* by using landscape phages immobilized on the quartz crystal. Vaughan et al., (2003) developed an on-site method for pathogen detection in fresh fruits and vegetables, using a phage-based ME biosensor. Ruan et al. (2004) observed *Staphylococcal* enterotoxin type B by using ME sensors.

19.4.3.10 Commercial Biosensor Transducers

A variety of biosensors have been developed for commercial applications in the food industry (Table 19.3) (Arora et al. 2011).

19.5 Bacteriophages

Bacteriophages (also known as phages) are classes of viruses that specifically infect bacteria. They are considered one of the most abundant naturally occurring biological entities and are massively diverse. Phages, of which there are many different types, can be found everywhere, including in water, food, and soil. They pose little direct threat to other species. Some phages have evolved to be specific to bacteria at the strain level, while others infect a much larger range of bacteria (Sulakvelidze and Kutter 2005; Sidhu 2005).

The phage was discovered by a British pathologist, Frederick William Twort, in 1915. However, he did not pursue his discovery. Felix Hubert d'Herelle, a French Canadian researcher, rediscovered the existence of these natural viruses in 1917 (Douglas 1975; Ackermann 2003; Sulakvelidze and Kutter 2005; Gervais 2007). Since then, several detailed studies have been devoted to the mechanisms of phage

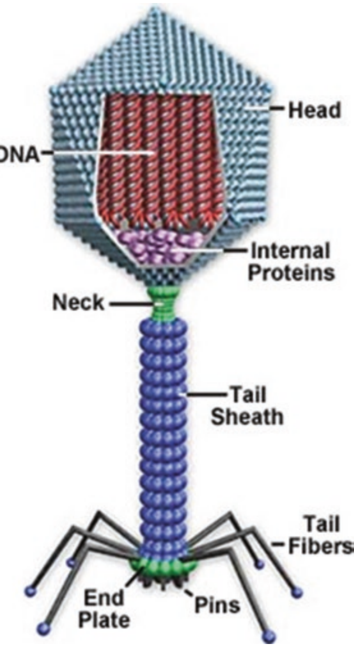

Fig. 19.4 Bacteriophage structure. (See webpage: www.micro.magnet.fsu.edu)

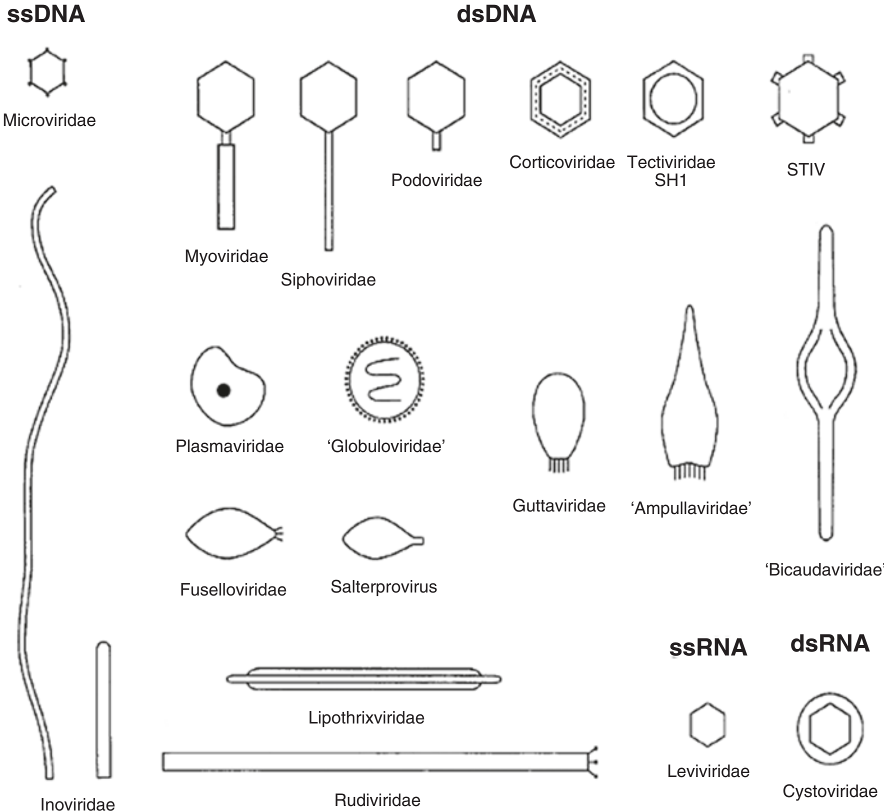

Fig. 19.5 Various phage morphotypes. (Ackermann 2009)

infection and reproduction (Ackermann 2003; Sulakvelidze and Kutter 2005). Electron microscopic findings have explained why some natural water supplies had antibacterial properties; these findings have also explained several other microbiological mysteries. Phages are robust viruses that are stable enough to infect bacteria often a decade after the phage has been assembled (Sulakvelidze and Kutter 2005).

Phages are complex macromolecules consisting mostly of proteins and genetic material (Fig. 19.4). Both DNA- and ribonucleic acid (RNA)-based phages exist (Fig. 19.5); however, DNA-based phages are more common. Phages are quiescent unless a host bacterium is present. Each phage is capable of infecting a bacterium and producing a large number of progeny; this process is called phage propagation (Sulakvelidze and Kutter 2005). Some phages have ten genes and depend almost entirely on bacterial cellular functions, whereas others have hundreds of genes and depend on proteins encoded by their own genetic material (Birge 1994). Primary research on bacteriophages focused more on their nature (Duckworth 1987; Wommack and Colwell 2000). Research has shown that phage proteins can be used as molecular vectors, for cloning, as diagnostic

and therapeutic agents, and for drug discovery (Loeffler et al. 2001; Smith et al. 2001; Schuch et al. 2002; Liu et al. 2004). Pettya et al. (2006) have described the biotechnological exploitation of bacteriophages. Virus electrodes for universal bio-detection (Yang et al. 2006) and phage-mediated biosensors have also been developed (Shabani et al. 2008; Tlili et al. 2013; Park et al. 2013)

19.5.1 Classification of Phages

Bacteriophage classification (Figs. 19.4 and 19.5) is based on nucleic acid type specificity, and structure (Luria et al. 1943; Nelson 2004). Phage structure has been classified by using electron microscopy. The molecular characterization of phages is done according to the types of nucleic acid present, i.e., single stranded (ss)-DNA, double stranded (ds)-DNA, and ss-RNA. DNA in bacteriophages can be either linear or circular (Fig. 19.5) (Thomas and Abelson 1966). The chromosome of a virus may account for up to 50% of its total mass (Birge 1994). Bradley (1967) and the International Committee on Taxonomy of Viruses proposed a scheme for classifying virus morphology and the nature of viral nucleic acids. Details of phage families are provided in Table 19.4 (Regenmortel 1990; Ackermann 2007, 2009). Phage chromosomes may be extremely small (such as the genome of *E. coli* phage R17, which is approximately 3600 bases in length and contains 4 genes) or relatively large (*E. coli* phage PB51, which is approximately 2.5×10^5 bases in length and contains 240 genes) (Birge 1994).

Table 19.4 Details of phage families (Ackermann 2009)

Structure	Nucleic acid	Family	Example
Tailed	dsDNA (linear)	*Myoviridae, Siphoviridae, Podoviridae*	T4
			Λ
			T7
Polyhedral	ssDNA (circular)	*Microviridae*	φX174
	dsDNA (circular, supercoiled)	*Corticoviridae*	PM2
	dsDNA (linear)	*Tectiviridae*	PRD1
	ssRNA (linear)	*Leviviridae*	MS2
	dsRNA (linear, multipartite)	*Cystoviridae*	φ6
Filamentous	ssDNA (circular)	*Inoviridae*	M13
	dsDNA (linear)	*Lipothrixviridae, Rudiviridae*	TTV1
			SIRV-1
	dsDNA (circular, supercoiled)	*Plasmaviridae, Fuselloviridae, Guttaviridae*	L2
			SSV1
			SNDV

ss Single stranded, *ds* double stranded

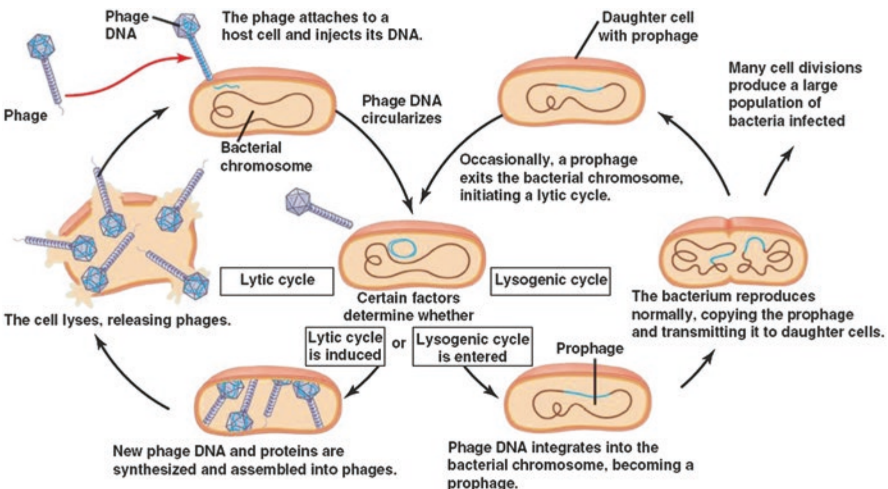

Fig. 19.6 Life cycle of bacteriophage (Ackermann 2009) (bio3400.nicerweb.net)

19.5.2 The Life Cycle of the Bacteriophage: Lysogeny and the Lytic Cycle

The first phase in the life cycle of phage infection is attachment to the host cell surface (bacterial surface). This is typically accomplished by the recognition of a receptor on the outside of the bacterial cell wall, such as an antigen, pilus, or other structure. There is much variability from phage to phage in terms of which receptor they bind to. Bacteriophages can generally be classified into two categories, lysogenic and lytic (virulent) (Fig. 19.6). The choice between the lytic and lysogenic cycles depends on the relative expression rates of phage repressors encoded by the *cII* gene (promoting lysogeny) and *cro* protein, which is capable of turning off repressor gene expression and starting the lytic pathway (Campbell 1967). The lysogenic cycle has been observed in dsDNA-containing phages; the phage DNA usually becomes part of the bacterial chromosome. These bacteriophage genomes will be replicated along with the genomes of the bacterial cells.

The lytic cycle involves the over-expression of phage proteins (Sulakvelidze and Kutter 2005). This happens sequentially, allowing phage assembly to take place. In tailed phages, this cycle often begins with the head and the tail proteins independently. Once the head is assembled, the phage DNA is packed in. The tail subsequently attaches to the head. Once this is complete smaller extremities are added to the tail; for example, in the T4 phage, its tail fibers are added. Phages remain in the bacteria as other phages are produced concurrently. Digestive enzymes encoded in the phage genome are eventually activated and transcribed, causing lysis of the host bacteria. This process releases the newly assembled phages into the environment, where each of them can infect a new host bacterial cell. T2, T4, and lambda phages are common lytic phages (Pelczar et al. 1988; Maloy et al. 1994; Gottesman and Oppenheim 1994).

Phages showing pseudolysogeny are in an unstable and inactive state. This occurs mainly when the host is exposed to starvation; when proper nutrients are added, this state resolves to true lysogeny (Williamson et al. 2001; Ripp and Miller 1997).

19.5.3 Bacteriophages Against Pathogens

Phages are very specific to host cells. Phages are found in environments such as waste water, fresh water, and soil (Kennedy and Bitton 1987). Bruttin and Brüssow (2005) reported that *E. coli*-specific phages were safe for oral administration in humans. O'Flynn et al. (2004) reported phage treatment of *E. coli* O157:H7-contaminated beef. Bacteriophages are used for biocontrol and against pathogens (Sheng et al. 2006; Wagenaar et al. 2005; Fiorentin et al. 2005).

19.5.3.1 Bacteriophage Treatment of Toxinogenic *E. coli*

E. coli causes a variety of human illnesses, such as abdominal cramps, bloody diarrhea, and vomiting. O'Flynn et al. (2004), in their report on phage treatment of *E. coli* O157:H7-contaminated beef, used different phages as biocontrol agents to eliminate the pathogenic bacteria in the contaminated beef. Raya et al. (2006) reported that T4-like and T5-like bacteriophages reduced intestinal *E. coli* levels.

19.5.3.2 Bacteriophage Treatment of *Campylobacter*

Campylobacter are frequently responsible for human disease, with very serious outcomes. *Campylobacter* cause oral infections, and, in industrialized nations, they also cause foodborne diseases that arise from the consumption of contaminated poultry products (Loc Carrillo et al. 2005). Recent studies have focused on bacteriophage therapy to reduce *C. jejuni* colonization of broiler chickens, limiting the entry of these pathogens into the food chain (Wagenaar et al. 2005). Goode et al. (2003) reported a reduction of experimental *C. jejuni* contamination of chicken skin by using bacteriophages.

19.5.3.3 Bacteriophage Treatment of *Salmonella*

Salmonella infection is a major public health burden, as it causes food-related illnesses. Atterbury et al. (2007) reported that bacteriophage therapy reduced *Salmonella* infection. Goode et al. (2003) observed a reduction of experimental *Salmonella* contamination of chicken skin by using bacteriophages. Whichard et al. (2003) reported the bacteriophage-induced suppression of *Salmonella* growth in a broad host range.

19.5.3.4 Bacteriophages to Control *Listeria* Contamination

Listeria monocytogenes infection may account for the lowest incidence of foodborne infections, but it is a serious threat to human health as an opportunistic pathogen in food. Guenther et al. (2009) reported a bacteriophage for the effective biocontrol of *Listeria monocytogenes* in ready-to-eat foods. On fresh-cut produce, biocontrol of *Listeria* was achieved by treatment with lytic bacteriophages, both

alone and in combination with a bacteriocin (Leverentz et al. 2003, 2004). Phages have potential and versatility as agents for the biocontrol of *Listeria* (Hagens and Loessner 2007).

19.6 Isolation of Phages Against Foodborne Pathogens

Pathogenic bacteria with antibiotic resistance have become a significant public health hazard, in particular to elderly, young, and immunocompromised individuals. Phage research is now focused on the infection of pathogenic bacteria—such as *E. coli*, *Campylobacter*, *Salmonella*, *Listeria*, and *Streptococcus*—by phages that have been isolated and characterized from different environmental samples; each of these phages can be used as biocontrol agents. *E. coli* phages have been isolated from fresh chicken, beef, mushrooms, vegetables, and packaged food, with counts as high as 10^4 phages per gram of sample (Allwood et al. 2004). Atterbury et al. (2003) reported that *Campylobacter* phages have also been isolated from chicken, at levels of 4×10^6 plaque-forming units (pfu).

E. coli is the most commonly isolated enterobacter species and is the main etiological agent of gastrointestinal infection. EHEC strains, such as *E. coli* O157: H7, are found mostly in ruminants. The EHEC strains of *E. coli* cause heavy bloody diarrhea and HUS. Many procedures have been reported for the isolation of phages of *E. coli*, but generally these have had limited success (Begum et al. 2010; Mahony et al. 2011). An improved isolation procedure for *E. coli* phages has been reported; this was an enrichment method for isolating the phages with potential host strains (Smith and Huggins 1982, 1983; Smith et al. 1987; Loessner et al. 1993; Jamalludeen et al. 2009); with enrichment methods, 43 phages against a number of *E. coli* and *C. jejuni* strains were isolated, revealing their host range and enhancing food safety. Phages against foodborne pathogens, such as *Streptococcus suis* and *S. aureus*, have also been isolated (Ma and Lu 2008; Synnott et al. 2009). Phages that are capable of infecting *Salmonella* strains associated with foodborne illnesses have also been isolated (Callaway et al. 2010). These phages are used as biotherapeutic agents and as pre-harvest biocontrol agents.

You and Yin (1999) reported that viruses were amplified during plaque growth in a reaction diffusion system. Jamalludeen et al. (2009) isolated and characterized a complete set of phages that were active against *E. coli* serogroups O1, O2, and O78. Jamalludeen et al. (2007) reported isolated and characterized phages that could be used in the prevention and treatment of porcine post-weaning diarrhea caused by O149 enterotoxigenic *E. coli* (ETEC).

Loessner and Bussesse (1990) reported the typing of bacteriophages against *Listeria* obtained from different dairy products and other food products. Tartera and Jofre (1987) reported 12 strains of different *Bacteroides* species that were tested for their efficiency of bacteriophage detection in sewage. Grabow (2001) reported on the fundamental properties and features of phages. Sword and Pickett (1961)

studied bacteriophages of *L. monocytogenes* in regard to isolation techniques and their use as diagnostic tools and as aids in epidemiological investigations. Twarog and Blouse (1968) isolated and characterized transducing bacteriophage BP1 for *Bacterium anitratum (Achromobacter sp*.). The particle had a head dimension of 450 A° and a tail approximately 200A° long. Sharpy et al. (1986) reported the isolation of 24 thermophilic bacteriophages from different natural sources such as compost waste, soil, silage, and rotting straw; the phages were able to infect most samples of *B. thermophilus*. Grabow et al. (1984) reported plaque assays using *E. coli*. Kudva et al. (1999) reported the isolation of an *E. coli* O157 antigen-specific bacteriophage. Begum et al. (2010) reported isolated phages that were specific for ETEC virulence factors. Kropinski et al. (2013) reported bacteriophages used as analytical tools to control foodborne pathogens in foods and in animals. Akhtar et al. (2014) purified phages isolated from animal feces and sewage samples, characterized the phages morphologically and by DNA fingerprinting, and determined their host ranges.

Loessner et al. (1993) isolated, classified, and characterized bacteriophages for *Enterobacter* species. Buchwald et al. (1970a, b) reported the morphogenesis of a lambda (λ) bacteriophage and identified the principal structural proteins. Analytical separation of the proteins of the λ bacteriophage was done by treatment with sodium dodecyl sulfate (SDS) at neutral pH and high temperature, followed by electrophoresis in acrylamide gels containing SDS. Laemmli (1970) reported gel electrophoresis in which unknown proteins in bacteriophage T4 were measured.

Tanji et al. (2005) reported the therapeutic use of a phage in controlling *E. coli* 0157:H7, which is associated with hemorrhagic colitis.

Singh et al. (2016), using an overlay method, reported the isolation of a phage against *E. coli* from waste water samples; the phage titer was about 10^7 pfu/ml. Stability was determined for different parameters, e.g., pH, temperature, and ultraviolet (UV) radiation. The phage morphology was determined by transmission electron microscopy (TEM). The phage capsid was about 78 nm in diameter, with a tail length of 527 nm, as compared with the wild-type λ phage, whose head is about 65 nm. The isolated phage was classified as belonging to the order *Caudovirales* and family *Siphoviridae*. Molecular characterization of the isolated bacteriophage (dsDNA >33.5kbp) was carried out and compared with standard λ DNA by performing restriction enzyme digestion, using *BamHI*, *EcoRI*, and *HindIII*. The results of restriction digestion were compared with in-silco results and were found to be similar. Sodium dodecyl sulfate-polyacryamide gel electrophoresis (10% SDS-PAGE) profiles for protein structure analysis indicated ten protein bands of different molecular weight that were stained with Coomassie blue, followed by de-staining. It is therefore proposed that the isolated phage be classified as a λ-like virus. This phage could infect and kill several potentially harmful bacteria, e.g., *E. coli*, and could be used as a control agent. This isolated phage could be utilized as a biological component in the development of biosensors for the detection of foodborne pathogenic bacteria.

19.7 Phage-Based Detection Systems

Geng et al. (2008) reported an electrochemical sensor for the detection of *E. coli*; they used anti-*E. coli* antibodies on a gold electrode surface. The immobilization of antibodies and other bio-receptors at the gold, silver, or platinum electrode was carried out through a self-assembled monolayer (SAM) method. This electrochemical sensing system has a detection limit of 1.0×10^3 cfu/ml.

Tlili et al. (2013) reported employing a bacteriophage as the biological recognition element in label-free electrochemical impedance spectroscopy. Naidoo et al. (2012) reported purified bacteriophages for the optimized capture of bacteria. Tolba et al. (2012) developed a biosensor using a bacteriophage immobilized on a gold screen printed electrode, and they used electrochemical impedance spectroscopy (EIS) for the detection of *Listeria* cells.

Shabani et al. (2008), using a functional carbon electrode, found a bacteriophage used as a recognition receptor was able to detect specific bacteria. Mejri et al. (2010), using EIS, compared the use of a bacteriophage and antibody recognition material for the detection of specific bacteria.

Muñoz-Berbel et al. (2008) used impedance spectroscopy to quantify bacteria, specifically *E. coli*, immobilized on platinum surfaces. Dastider et al. (2015) reported a microfluidic chip to detect *Salmonella typhimurium*, using a monoclonal anti-*Salmonella* antibody recognition bio-receptor. Shabani et al. (2007) reported the immobilization of bacteriophage T4 on a carbon surface. Hengerer et al. (1999) and Uttenthaler et al. (2001) reported an immunosensing system based on a QCM. Pathirana et al. (2000) reported the Langmuir-Blodgett method used to immobilize antibody for the detection of *Salmonella typhymurium.* Vaughan et al. (2001) reported the development of a QCM immunosensor for the detection of *L. monocytogenes.* A thiosalicylic acid SAM was incorporated for the covalent attachment of antibodies to the gold surface of the piezoelectric crystal. Vaughan et al. (2003) reported a rapid, label-free QCM sensor for the specific detection of the *E. coli* pathogen. Dultsev et al. (2001) reported that the surface of a QCM could be used to detect a specifically adsorbed bacteriophage. Singh et al. (2015a) reported using the SAM method to immobilize bacteriophages on a gold surface for the detection of *E. coli*. In another study, Singh et al. (2015b) reported the detection of *E. coli* using a bacteriophage as a recognition bio-receptor, and measured using electrochemical impedance sensing.

Singh and Jain (2017) reported the development of a QCM sensor for the detection of *E. coli*. An electrochemical quartz crystal microbalance (EQCM) is a very sensitive device that measures the mass change per unit area by measuring the change in resonance frequency of a quartz crystal. A SAM of 11-mercaptoundecanoic acid (MUA)/1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC)/hydroxysuccinimide (NHS) was incorporated for the covalent attachment of the phage to the gold surface of the piezoelectric crystal. A Sauerbrey increase in frequency was observed upon the exposure of such a crystal surface, modified for a phage, to *E. coli* cells. The electrochemical cell was provided with a module

oscillation frequency of 6 MHz. The sensor detected *E. coli* cells in solution, in real time, to 1×10^5cfu/ml.

19.8 Bacteriophage Application in Biosensor Development

Biosensors are increasingly being used as an alternative to conventional methods for the detection of foodborne pathogens (Lazcka et al. 2007). A biosensor, a device for the detection of an analyte, combines a biological recognition component with a detector component. These biological components may include enzymes, antibodies, tissues, organelles, whole cells, DNA, or phages. The design of a biosensor requires the interaction of the analyte with the biological agent to be specific, selective, and stable for a long period of time. Affinity-based biosensing has used monoclonal or polyclonal antibodies for specific antigen recognition (Pancrazio et al. 1999). A phage-based biosensor is an attractive alternative to immunosensors, because phages are ever-present components of microbial communities on earth and are, therefore, easy to isolate (Shabani et al. 2007, 2008; Tlili et al. 2013). Bacteriophages exhibit faster binding and are cheaper and simpler to mass produce than antibodies; specific bacteriophages have been isolated that are able to infect either only certain species of bacteria or the whole genus (Goodridge and Griffiths 2002). The specificities of bacteriophages for their host bacteria make them ideal agents for bacterial identification and strain typing (Dubow 1994). Shabani et al. (2008), using a functional carbon electrode, reported a bacteriophage used as a recognition receptor to detect specific bacteria. Vaughan et al. (2003) reported a rapid, label-free QCM sensor for the specific detection of *Bacillus cereus*. The chemical attachment of T4 bacteriophages onto a gold surface has been reported by Gervais (2007). Biosensor techniques in the field of processing and quality supervision show advantages as alternatives to conventional methods, owing to their high sensitivity and specificity, rapid provision of results, and cost efficiency. Biosensor technology is very promising, but there are still technological problems to be deciphered. Additionally, market penetration has to be improved for areas where biosensor technologies are essential for elevating food diagnostics. As interest in safe food and water supply is increasing, the demand for biosensors that provide rapid results will also be boosted (Sharma et al. 2013).

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20 Computational Tools and Databases of Microbes and Its Bioprospecting for Sustainable Development

Dipannita Hazra and Atul Kumar Upadhyay

Abstract

The large diversity present in ecosystem has tremendous potential in the microbial bioprospecting. Microbial bioprospecting is a branch of science, which deals with the identification of suitable microorganisms, biological compounds, or gene sequences which can be used for useful compounds for human welfare. Computational approach to biology is one of the rapidly emerging and promising branches of science. In the past few years, there is dumping of enormous amount of biological data especially from microbial genomes and transcriptomes to public databases. To use these data for the improvement of quality and quantity of microbial products for sustainable development, one needs to expertize in computational methods. In this chapter we have discussed the computational tools (techniques and databases) for better understanding of microbial genes, genomes, and proteome. We have also discussed the importance and uses of next-generation sequencing (NGS) tools to understand microbial genetics and genomes for better production of microbial products such as antibiotics, fermented products, biofuels, etc. Application of these approaches, tools, techniques, and databases to understand the microbial genes, genomes, and proteome would have tremendous effect on development, improvement, and sustainable cultivation of microbes.

Keywords

Bioprospecting · Computational biology · Microbes · Genome · Proteome · Sequences

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20.1 Glimpses of Microbial Bioprospecting

The discovery and commercialization of valuable products based on biological resources have gained interest. The large diversity in the ecosystem has shown to have a greater potential in the bioprospecting. Bioprospecting deals with the search of organisms, biological compounds, or gene sequences which can be used for mankind, and it mainly consists of three steps:

1. Identification of source
2. Evaluating the source
3. Exploiting and screening of the source for commercial (or valuable) product

With the advances in the research and technology, the raw materials around us are being used as a source for renewable energy; that is why bioprospecting is also rightly known as biodiversity prospecting; thus it also means commercializing the biodiversity. Bioprospecting should mainly focus on three things: (i) conservation of the biological diversity, (ii) sustainable development, and (iii) sharing the benefits arising from it in the justifiable and fair manner, as it can be advantageous or disadvantageous depending on how well it is managed. If properly managed it can act as a revenue source for the developing countries, and can also help in developing new and novel compounds. On the other hand, if it is not managed properly, it can result in environmental, social, and economic problems. It may lead to disrespect of the rights of society and mankind (Bijoy 2007; Millum 2010). Sometimes it is seen that the developing countries are being exploited because of bioprospecting, and it sometimes can lead to over-exploitation of the biological diversity. Thus, it is very important to balance the growth of bioprospecting without harming the environment (Dhillion et al. 2002). The regulation of bioprospecting should be done in both national and international levels by following ethical ways, so that it does not affect the biodiversity. It should focus on sustainable development and develop strategies to improve the resources available. The success rate of bioprospecting has attracted researchers to focus on this area. Earlier it was only focusing on plant species in the ecosystem, but now with the advancement in research and technology, it is also focusing on other species like algae, microorganisms, etc.

Microorganisms are present everywhere and it has the ability to survive in extreme conditions. The biodiversity observed among the microorganisms is vast, and despite this, we know only 1% of the total biodiversity. The rest 99% of the microorganism are yet to be explored. The insufficient laboratory culturing techniques is one of the primary reasons for this issue (Akondi and Lakshmi 2013). Microorganisms are known to have wide range of capability, from causing diseases to different life forms to providing valuable products like antibiotics, immunosuppressants, enzymes, bioactive compounds, etc. They also help in bioremediation and biodegradation of organic waste materials. These microorganisms have shown a diverse role in the ecosystem and have given a new platform for research and development. They act as the reservoir for the synthesis of different novel and valuable product not only for the welfare of the society but also help the environment by

removing and detoxifying pollutants. The knowledge of growth rate, easy isolation techniques, and extraction of product (intracellular and extracellular) have made them more suitable for this application. Thus microbial bioprospecting is one of the important areas, which help us to move in a sustainable manner.

Use of microbes to produce valuable products is known from the ancient times. The discovery of penicillin is one such example, which was widely used as an antibacterial compound during the Second World War. Using the same technology, various naturally occurring compounds like streptomycin, erythromycin, etc. were also developed (Demain and Sanchez 2009). Majority of the natural products obtained from the microbes are being used directly without any significant modifications, while some requires different chemical modification prior to use. Endophytic microbes have gained interest as they are found on most of the plants in the ecosystem. They are present in the living tissue of their host and have shown a variety of relationships from symbiotic to pathogenicity. Thus, they have been exploited as the potential source for producing novel compounds, which have significance in agriculture, medicines, and industrial benefits. It is considered that endophytes have genetic diversity for various novel traits, which is very reliable. Most of the genes of these species are unannotated, and thus we need to focus our research toward this area for better understanding of these genes (Dreyfuss and Chapela 1994). Different studies have been done in order to develop recombinant pharmaceuticals from the microbes. The most used organisms to develop recombinant pharmaceuticals are *Escherichia coli and Saccharomyces cerevisiae*. In a study *E. coli* and *S. cerevisiae* have been used for the protein drug production (Ferrer-Miralles et al. 2009). Human diseases like diabetes, clotting disorders, etc. are related to protein disorders. These diseases require administration of functional proteins, which are synthesized ex vivo. By using recombinant DNA technology, different microorganisms have been used to produce therapeutic protein (Sanchez-Garcia et al. 2016).

Some studies have shown that there are genes, which remain silent under the laboratory techniques. These genes can be responsible for providing more diverse secondary metabolites. For further advancement in the drug discovery, the microbial communities can be targeted. These communities interact by using different signals or defensive mechanisms, which can be explored for novel, compound synthesis. Advanced analytical methods such as mass spectrometry, metabolomics, etc. have been used in order to understand the induced metabolites, the chemical diversity, and the biological diversity of the microorganism co-cultures (Bertrand et al. 2014). With the increase in the demand of food, reduced use of chemicals has significantly focused the researchers to identify organic or natural products to protect the crops from the pathogens. Many soilborne fungi are responsible for damaging the important crops, and it is the major concern of the agriculture food production. The microorganisms producing mycolytic enzyme have proved to play a significant role in this. These microorganisms have the ability to lyse the cell wall of fungi and at the same time convert the chitinous waste into an enzyme chitinase, which helps in protecting the host from fungal pathogens. Various studies have showed that chitinase gene has been successfully transformed into many plants (Gohel et al. 2006). Approximately 15 *Penicillium* strains

have been isolated and tested to have antifungal properties, and further studies have shown that 12 of these strains have antitumor properties. This study has shown that if the fungal extracts are directly assayed on the tumor cells, it restricts the work required to attribute the bioactive molecules to a reduced number for important strains (Nicoletti et al. 2008).

Marine microbes are also considered as the potential source for the production of novel bioactive compounds. The well-known class of bacteria, actinomycetes, is said to produce different types of chemical metabolites, which have wide range of biological applications (Purves et al. 2016). It has been observed that the secondary metabolites released by these microbes have great significance in drug discovery. Bioactive compounds obtained from these marine microbes have shown to be of great importance in the biotechnology and pharmaceutical applications (Zhang et al. 2005). In a study it was observed that Manikaran hot spring has thermotolerant bacteria, which produce hydrolytic enzymes. 120 different strains were isolated. Twenty of them showed hydrolytic enzyme production at temperature below 70 ° C, and seven of these strains showed novel and valuable production of hydrolytic enzyme. Some organisms produce proteins that have the ability to control the ice crystal formation. Studies have shown that these proteins provide an advantage to the species during the phase change due to the temperature. The proteins have been isolated and are being used in various research and industrial applications for cryopreservation, food preservation and preparation, etc. (Christner 2010). Sponges are the marine organisms which have shown a wide range of bioactive compounds. However not all of these are being produced by them, but rather it is produced by the bacteria and fungi associated with sponges. The test proved that antimicrobial substances are being released by these sponge-associated microbes which include HIV-1, influenza A virus, etc. In this study 35 different bacterial and 12 fungal genera were identified that can produce antimicrobial compounds (Indraningrat et al. 2016). Soil metagenome is another focused area in research. Most of the soil microbes have not been characterized yet, and thus the major focus lies on the development of the culture techniques. With the help of high-throughput screening technologies, some very important and novel enzymes have been isolated from the soil, e.g., lipolytic enzymes. The most prevailing and important enzymes found were esterase and lipases. They have significant applications as biocatalyst in various biotechnology industries. Apart from this there are other enzymes and important bioactive compounds, which have also been isolated from soil metagenome (Lee and Lee 2013).

Over the last few years, studies have been done in order to screen and cultivate microorganisms with biotechnology potential. Sequence-based metagenomics can identify numerous genes present in the sequence which can encode for significant enzymes, but it is hard to consider that all these genes can be expressed as active enzymes in the available hosts. Apart from this, the isolation of microorganism present in the extreme conditions often acts as a challenge in bioprospecting because of various factors like low cell biomass, poor cell growth capacity, restricted environment access, etc. Some studies and different approaches have been done in order

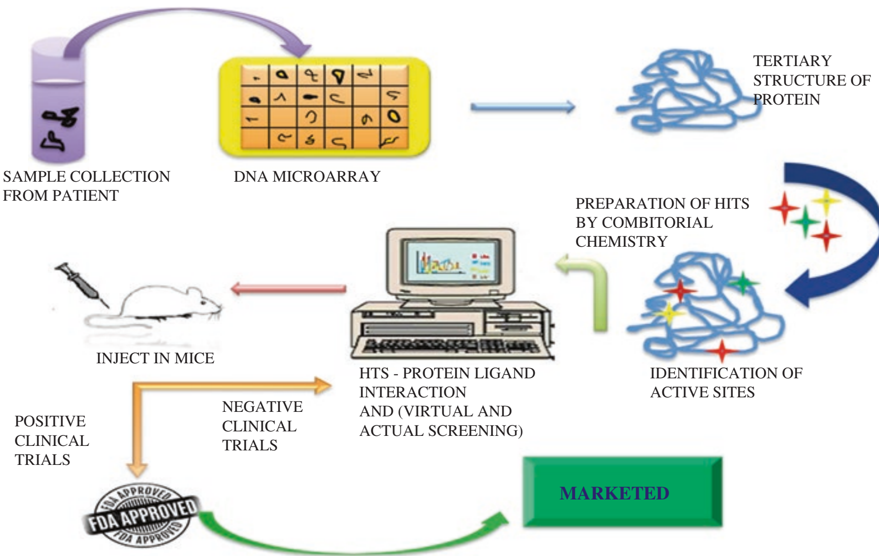

Fig. 20.1 Cartoon representation of steps of drug discovery (bioprospecting) starting from sample collection to processing of the data and analysis. Involvement of computational tool is almost at all the step shown with the arrows in the figure

to improve the isolation and culturing techniques faced by the bioprospecting of microorganisms in cold environment. Bringing the natural environment in the laboratory and then cultivating the microorganisms is found to be advantageous. Few of the methods like diffusion chamber and iChip which is a novel approach for simultaneous cultivation and isolation of uncultured microbes (Kaeberlein et al. 2002), hollow-fiber membrane chambers (Aoi et al. 2009) which help in maintaining the perfect environment, and gel microdroplets and I-tip which is recently developed (Zengler et al. 2002) can be used directly in the cold environment for the cultivation of the microorganisms. The growth condition difficulty and media composition can be a problem for these if it is being used in hostile environment or in remote locations (Vester et al. 2015).

Another method that is being used for bioprospecting is computational approach, in which the genome sequences already present in the database are searched for novel genes, enzymes, or pathways for its application in industry or research. This method of comparing and analyzing the evolutionary relationship in the genome sequence is fast and cost-effective; thus its application is increasing in bioprospecting. The faster method of characterizing and identifying different sites present in the protein obtained from the diseased patient has made it easier to prepare drugs by identifying different ligand interactions.

Studies are done in order to develop new drugs by lead preparation and performing synergy screening with the already existing natural or synthetic approved drugs and bioactive compounds in the database (Fig. 20.1). Antituberculosis leads have

already been manufactured from the microbial metabolites (Ashforth et al. 2010). The improvement in the technology has allowed rapid sequencing, characterization, and analysis of the whole genome sequences of bacterial and fungal species. This has provided us with a hub in the form of gene cluster, which serves as the potential source for novel biological compound synthesis (Zotchev et al. 2012).

20.2 Databases and Computational Tools to Study Microbes and Its Products

These approaches and their use for biological problems are also known as "bioinformatics," which helps in better comprehension of biological systems. In order to understand the biology of microbes, a detailed understanding of their genes, genome, proteins, proteome, and transcriptome is required. There are different set of computational tools to study these molecules and their interactions. Bioinformatics methods are very useful and prominent tool to perform analysis of large number of datasets to provide an early understanding and screening of interesting targets for detailed experimental characterization. For example, one of the most used tools is BLAST (McGinnis and Madden 2004), which can search homologue protein or DNA sequences for a given query protein or DNA sequence in sequence databases. These databases contain millions of entries, and it is practically impossible to perform similarity searches manually in such databases. There are dedicated databases for DNA, protein, pathways, metabolites, etc. In the last decade or so, the use of high-throughput experimental techniques at larger scale in system biology has generated un-comparable amount of data. It is now almost unavoidable and very crucial to use computational means to gain further insights in the field of system biology. In this chapter, many day-to-day used bioinformatics tools and resources are discussed. These tools would be used to understand the development and sustainable growth of microbes and their products in detail.

Genes are made up of DNA and their structures are not so complex as compared to proteins. Proteins are translational product of genes, which is made up of amino acids and acquires a complex structure categorized into class, fold, superfamily, family, etc. (Jones and Thornton 1995). Properly folded protein molecules govern molecular functions of a system. Protein folding is a spontaneous process within the cell. Protein folding depends on several factors like pH, temperature, and concentration of proteins. Following is list and short description of some of the widely used tools and techniques in bioinformatics for analysis of biomolecules such as DNA, protein, mRNA, and pathways. As the genomes of the microbes are much smaller compared to eukaryotes, there is different and specialized set of tools, software, and techniques for microbial data analysis (Table 20.1).

There are several important and useful tools and databases for the analysis of microbial genes, proteins, pathways, etc. for its efficient bioprospecting. Detailed explanation of few of these databases and tools is discussed in the following sections.

Table 20.1 List of important databases and servers for the analysis of genome, proteome, and pathways of microbes

S. No.	List of software	Link
1	MicrobesOnline	http://www.microbesonline.org/
2	metaMicrobesOnline	http://meta.MicrobesOnline.org
3	MicrobesFlux	http://tanglab.engineering.wustl.edu/static/MicrobesFlux.html
4	MicrobeGPS	https://sourceforge.net/projects/microbegps
5	PSORTdb	http://db.psort.org
6	BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
7	PSI-BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
8	Pfam	http://pfam.xfam.org/
9	PDB	https://www.rcsb.org/pdb/home/home.do
10	Modeller	https://salilab.org/modeller/
11	EuMicrobDB	http://www.eumicrobedb.org/eumicrobedb/index.php
12	MicrobeCensus	https://github.com/snayfach/MicrobeCensus
13	iMicrobe	https://www.imicrobe.us/
14	AgBase	http://www.agbase.msstate.edu/
15	ProTraits	http://protraits.irb.hr/
16	Vikodac	http://metagenomics.atc.tcs.com/vikodak/
17	GLAMM	http://www.microbesonline.org/cgi-bin/glamm
18	RevEcoR	https://cran.r-project.org/web/packages/RevEcoR/
19	MOST	https://github.com/thuangsh/most
20	HPMCD	http://www.hpmcd.org/

20.2.1 MicrobesOnline

MicrobesOnline (Alm et al. 2005) is an online tool for annotation of microbial genes. It has more than 1000 complete genomes of microbes of different taxon, viz., bacteria, archaea, and fungi. Along with the gene information, this server also harbors expression profiles of thousands of mRNA from many diverse organisms. MicrobesOnline also has a genome browser, which helps in comparing genomes or gene families on the basis of phylogenetic trees for every gene family as well as a species tree.

20.2.2 metaMicrobesOnline

The metaMicrobesOnline database "http://meta.MicrobesOnline.org" (Chivian et al. 2013) helps in performing phylogenetic analysis of genes from microbial genomes and metagenomes. Most of the gene trees are for the canonical gene families, e.g., form Pfam and COG. In this database a genome browser is also imbedded, which allows genome comparisons of microbes. Other interesting feature of the database is that the browser allows comparison of protein domain organization of the genes from different genomes and metagenomes. The structure of this database

Table 20.2 Tabular representation of number of genomes and metagenomes under different sections in metaMicrobesOnline database

S. No.	Category	Numbers	Remark
1	Microbial isolates	1629	1429 are bacterial, +80 are archaeal, +120 are eukaryotic fungal and algal
2	Metagenomes	155	123 ecological and 32 organismal associated
3.	Cluster of orthologous groups	4873	Describes number of orthologous gene groups
4.	Pfam domain family	12,148	Number of domain families

is represented in tabular form (Table 20.2). There are approximately seven million genes in this database.

20.2.3 MicrobesFlux

MicrobesFlux is a web server for studying metabolic pathway models of diverse microbes (Feng et al. 2012). This server builds and modifies according to several parameters and analyzes the metabolic models. To generate models of metabolic networks, this server uses LIGAND database along with KGML files from KEGG database. MicrobesFlux is available at "http://tanglab.engineering.wustl.edu/static/MicrobesFlux.html" and is supported by several web browsers such as Google Chrome, Mozilla Firefox, and Safari. MicrobesFlux has three components, viz., logic, application, and achievement.

20.2.4 MicrobeGPS

This tool is of great use in assigning relatedness among the strains of microbes in a microbiota sample. It calculates the genomic distances and identifies the closest reference genome. MicrobeGPS (Lindner and Renard 2015) has the ability to resolve the genomes at strain level. It is freely available and the source code can be accessed at the given link: https://sourceforge.net/projects/microbegps. The binary for Windows and Linux is also available on this site. SAM files of the reads mapped to the reference genomes are used as input to the MicrobeGPS. It analyzes the SAM files after filtering the reads. It calculates a score and sequencing depth for each reference genome to identify the related genomes. MicrobeGPS performs clustering of related genomes in different groups; each group is unique biological sample.

20.2.5 PSORTdb

PSORTdb (Peabody et al. 2016) is a database of information of protein subcellular localization (SCL), which is an essential parameter for understanding protein function. SCL also helps in genome annotation and have various other applications such

Table 20.3 List of variants of BLAST and description of query-database type to search in the respective variant searches

S. No.	Variants of BLAST	Query sequence	Database type
1	BLASTN	Nucleotide	Nucleotide
2	BLASTP	Protein	Protein
3	tBLASTn	Protein	(Translated) nucleotide
4	BLASTx	(Translated) nucleotide	Protein

as diagnosis of drug targets. PSORTdb is freely available at "http://db.psort.org." The experimentally verified information about subcellular localization of proteins is kept in other repository known as ePSORTdb. Latest release of it is PSORTdb 3.0, which is user-friendly and has information of protein SCL of difficult entries (non-classical bacterial proteins).

20.2.6 MOST (MOst Similar Ligand-Based Target)

It is a web server for the prediction of targets of ligand compounds by using fingerprint similarity. It also tells the bioactivity of the ligands. Evaluation of the performance of MOST (Huang et al. 2017) is done by various methods such as machine learning, fingerprint schemes, etc. Selection of target is a most important element to understand the molecular mechanism of action of chemical or herbal compounds. It is freely available at https://github.com/thuangsh/most.

20.2.7 BLAST

Assigning relationship to a given sequence of protein or DNA by searching homologous sequences in the public databases is one of the basic and important parts of bioinformatics analysis. Basic Local Alignment Search Tool (BLAST) (McGinnis and Madden 2004) is one of the most widely used tools for this job. This tool comes in many variants such as BLASTP, BLASTN, tBLASTn, and BLASTx. Description of these variants is provided in tabular form (Table 20.3). In BLAST, different parameters such as e-value, bits score, query coverage, and similarity percentage could be adjusted.

A. Procedure to perform a general BLAST search:
   - Open the NCBI database on any browser.
   - Go to BLAST home page of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
   - Select the BLAST variant based on your query and target database such as BLASTP for protein sequence query against protein nr database.
   - Explore the different input options (e.g., type of BLAST, database option, scoring scheme, etc.)
   - Perform protein BLAST with default parameters if you do not have specific requirement.

- Analysis of the output file based on different parameters such as alignment score, e-value, identity, gaps, etc.
- Change parameters, e.g., BLOSUM, e-value cutoff, masking option, etc. Observe influence on output.
- For the same query protein, compare the protein sequence as well as coding nucleotide sequence across database. Compare the results.

20.2.8 PSI-BLAST

PSI-BLAST is Position-Specific Iterative-BLAST, which first searches homologous sequences as normal BLAST and then creates PSSM (position-specific scoring matrix) profile of these homologous sequences (Altschul et al. 1997). Later these PSSM profiles are used as separate queries to search in the database. The matrix is further used to validate and score the hits obtained in subsequent iterations. Different search parameters are present which could be adjusted according to the need of user.

20.2.9 The Pfam Protein Families Database

Protein domains are structural and functional unit of proteins. On the basis of protein domains, proteins are classified into different families and stored in the form of database known as Pfam database (Finn et al. 2013). Each family in this database has a well-curated multiple sequence alignment, seed sequences (best representative sequences) (Joseph et al. 2014) for each family along with a hidden Markov model (HMM) of all the members of that family. Pfam database has two important categories Pfam-A and Pfam-B. Pfam-A entries are manually curated and checked families with representative high quality multiple sequence alignments whereas Pfam-B is for obsolete families.

20.2.10 Protein Data Bank (PDB)

The PDB is database of the tertiary and quarterly structures of biological molecules, prominently proteins and fewer molecules of nucleic acids (Bernstein et al. 1977). Majority of the structural data in this database comes from X-ray crystallography or NMR spectroscopy. The PDB is a very useful repository, freely available to everyone just a clicks distance. Structural biologist uses this database on regular basis. Some of the derived databases of PDB are SCOP (Murzin et al. 1995) and CATH (Knudsen and Wiuf 2010). At present PDB has nearly 1 lakh 34 thousand structures, out of which approximately 1 lakh 22 thousand structures are of proteins, 3 thousand structures are of nucleic acid, and around 6 thousand structures are of protein-nucleic acid complexes. Majority of the structures are determined by X-ray crystallography (~90%) method followed by NMR (9%), and the rest of the 1% structures are from electron microscopy and other methods.

20.2.11 Protein Structure Modeling by Molecular Modeling (Modeller)

Molecular modeling is a computational method of determining protein structure by taking a reference structure as template. Protein molecules with a sequence identity of 40% or more are referred as closely related protein sequences. In the twilight zone (sequence identity is less than 25%), alignment of template and target sequences becomes difficult. The first and crucial step of molecular modeling is identification of a template structure homologous to the target sequence. There are several databases and tools that have to be referred from comparative modeling such as PDB (Bernstein et al. 1977) and CLUSTALW (Larkin et al. 2007). Searching of template structure can be done by performing homologous structure search with the help of BLAST against structural databases such as Protein Data Bank (PDB) and Structural Classification of Proteins (SCOP) (Murzin et al. 1995) using query sequence. After successful, good-quality target template sequence alignment, Modeller software (Sali and Blundell 1993) could be used for the generation of 3D model of the target protein sequence.

20.3 Conclusion

The population of our world is increasing continuously and so are our demands. This increase in demand has resulted in the disruption of balance in the ecosystem, which has significant effect on the environment. The accumulation of pollutants, soil infertility, releasing of toxins in the water bodies, diseases to life forms, etc. are all such problems which have hampered the balance. The big challenge now is to have sustainable development that should not affect the environment or the future generation. Thus, bioprospecting provides us with a platform for sustainable development. It has provided a path and new area to focus our research in order to maintain the proper balance in the nature.

Computational approach is another method of bioprospecting, in which the gene and protein sequences present in the public databases are searched for novel genes, enzymes, or pathways for its application in industry or research (Upadhyay et al. 2015). Usage of computational methods in comparing and analyzing the evolutionary relationship in the gene/genome sequence is fast and cost-effective. It provides great opportunity to increase in microbial bioprospecting for sustainable development. The faster method of characterizing and identifying different sites present in the protein obtained from the diseased patient has made it easier to prepare drugs by identifying different ligand interactions.

A major step of efficient bioprospecting is to identify genes responsible for the productions of the compound. Once the responsible genes are identified, next step would be analysis of pathways in which these genes are involved for the production of the desired compound. Separately, finding the gene products in terms of proteins, its interacting partners, and structures needs to be performed to get a thorough knowledge of the regulation of the production of the compound. All these analyses

require a deeper understanding of bioinformatics tools and techniques. It is highly cumbersome to elucidate structures of all these sequences and assign their functions by experimental methods, leading to computational approaches. These methods exploit sequence information for automatic annotation transfer to hypothetical sequences.

In this book chapter, we have emphasized on the application of various computational tools and databases such as MicrobesOnline, metaMicrobesOnline, PSORTdb, MOST, BLAST, Pfam, PDB, etc. for the research and industry work on microbial bioprospecting. These tools, web servers, and databases are very useful to derive relationship among them to have better understanding of the molecular mechanism of production of microbial compounds along with genes, proteins, and pathways.

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