Vipin Chandra Kalia *Editor*

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Dedicated to My Parents

Preface

Plants, humans, and microbes show a strong interaction among themselves. Plants depend upon microbes for their growth and development and for acquiring nutrients. Plants in turn serve as food for human and animals. Microbes in the rhizosphere produce secondary metabolites to protect plants against pathogens and tolerate stress. The human skin and gut harbor a wide range of microbes, which are responsible for their well-being. This interaction of living beings is gaining renewed interest and value. Microbial activities are quite unique and interesting and are finding wider applications ranging from bioremediation, bioenergy, biomedicine, agriculture, and industry. During the past century, there has been a transition from chemical processes to biological methods, largely because the latter are eco-friendly. Now, the emphasis has shifted from only eco-friendly bio-processes to economical as well. The Green Technologies are the new trend to save the planet Earth through sustainable processes. Scientific progress can be gauged primarily through basic scientific research. As most scientific works are supported through public funds, there is a constant demand to put these findings to applications for human welfare. Students—the rising stars and our scientists in the making—are curious to learn the basic sciences and how these can be translated into products. This book has been brought out to cater to curious young minds and provide economic benefits to the society. In this book, the learned scientific community has put their best efforts to share their expertise, which they have gained through their immense experience targeted toward understanding the microbial world. This book is a true reflection of the sincerity of the scientific community, who promptly agreed to contribute their creation for the young minds, who are likely to benefit and take this world a step further into the future. I am truly humbled by the help rendered by the contributing authors. I am indebted to all of them. I am running short of words to adequately acknowledge the worthiness of their efforts. My true inspirations to write this piece of work stem from the faith in me and the constant support of the following individuals: the late Mrs. Kanta Kalia and Mr. R.B. Kalia (parents); Amita (wife); Sunita and Sangeeta (sisters); Ravi, Vinod, and Satyendra (brothers); Daksh and Bhrigu (sons); my teachers especially Dr. A.P. Joshi; and my

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About the Editor

Vipin Chandra Kalia is presently working as Emeritus Scientist. He has been the Chief Scientist, and the Deputy Director, at Microbial Biotechnology and Genomics, CSIR-Institute of Genomics and Integrative Biology, Delhi. He is a Professor of Academy of Scientific and Innovative Research (AcSIR), Delhi. He obtained his M.Sc. and Ph.D. degrees in Genetics from the Indian Agricultural Research Institute, New Delhi. He has been elected as: (1) Fellow of the Association of Microbiologists of India (FAMI), (2) Fellow of the National Academy of Sciences (FNASc), and (3) Fellow of National Academy of agricultural Sciences (FNAAS). His main areas of research are microbial biodiversity, genomics, and evolution, bioenergy, biopolymers, antimicrobials, quorum sensing, and quorum quenching. He has published 101 papers in scientific journals such as (1) Nature Biotechnology, (2) Biotechnology Advances, (3) Trends in Biotechnology, (4) Critical Reviews in Microbiology, (5) Bioresource Technology, (6) International Journal of Hydrogen Energy, (7) PLoS ONE, (8) BMC Genomics, (9) Gene, and (10) Annual Review of Microbiology. His works have been cited 3558 times with a h index of 31 and an i10 index of 66. He has edited 3 books: Quorum sensing versus quorum quenching: A battle with no end in sight (2015, Springer India), Microbial Factories Vol 1 and 2 (2015). He is presently the editor in chief of the Indian Journal of Microbiology and Editor of (1) PLoS ONE, (2) Journal of Microbiology & Biotechnology (Korea), (3) Applied Biochemistry & Biotechnology (USA), (4) International Scholarly Research Notices (Energy), (5) Dataset Papers in Science (Microbiology), and (6) Journal of Molecular and Genetic Medicine. He is a life member of the following scientific societies: (1) Society of Biological Chemists of India (2) Society for Plant Biochemistry and Biotechnology, India; (3) Association of Microbiologists of India; (4) Indian Science Congress Association; (5) BioEnergy Society of India, and (6) the Biotech Research Society of India (BRSI). He is also a member of the American Society for Microbiology.

Part I
Biomedicine

Role of Bacteria in Nanocompound Formation and Their Application in Medical

Rubbel Singla, Anika Guliani, Avnesh Kumari, and Sudesh Kumar Yadav

Abstract Nanotechnology has now reached to a stage where the nanoparticles (NPs) have been in applicability in wide-ranging realms of science and technology. NPs are the materials with at least one dimension in the order of 100 nm or less. NPs display astonishing properties of high surface/volume ratio and enhanced physical, chemical, optical, and thermal properties which are extremely different than their bulk materials. The conventional methods of synthesis of nanocompounds involve the employment of physical and chemical methods, which have few drawbacks such as the requirement of toxic hazardous chemicals, energy intensive, and costly processes make it difficult to be widely implemented. To overcome these limitations, the researchers have looked forward for an easy and feasible alternative approach for the synthesis of nanocompounds. The employment of alternative biogenic route for the NP synthesis by using biological entities of unicellular living organisms such as bacteria, fungi, and actinomycetes has sought apparent attention of the scientists throughout the global earth. A greener approach interconnecting nanobiology with microbial biotechnology is responsible for the formation of NPs mediated by microbes that allow synthesis in aqueous environment, with low energy consumption and at low costs. Biosynthesis of gold, silver, copper, quantum dots, and magnetite NPs by bacteria, fungi, actinomycetes, and yeasts has been reported. In a view to form noble metal NPs of uniform shape and size, biological routes using microbial cultures at optimal temperature, pressure, and pH have been

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formulated. In this chapter, the main focus is given on the intracellular and extracellular approaches used for synthesis of metallic NPs by various microbial species. A detailed discussion is provided to explain the various factors which affect the synthesis of nanocompounds to further augment the growth rate of NPs as well as the mechanism of action at the cellular, biochemical, and molecular level. A great stress is given on the role of these nanocompounds in the medical field for the diagnostic and disease treatment. The potential of great biodiversity of microbial cultures as biological candidates leading to the manufacturing of NPs is needed to be fully investigated.

Keywords Microbial synthesis of nanocompounds • Metal nanoparticles • Theranostics • Anti-microbial agents • Disease therapy

1 Introduction

The term "nano" has its origin from the Greek language meaning of miniaturized objects of one billionth (10^{-9}) in size. Nanoparticles (NPs) are the particles having at least one dimension in the nanometer scale, i.e., $\leq$100 nm. Nanocompounds have attracted a great attention due to their characteristic unique electronic, optical, physical, chemical, electrical, mechanical, magnetic, thermal, dielectric, and biological properties which are absolutely different from their bulk counterparts (Jain et al. 2008; Kato 2011). The metal NPs have an upper hand when compared to those metal compounds at molecular level due to their enhanced Rayleigh scattering, surface plasmon resonance (SPR), surface-enhanced Raman scattering (SERS) in metal NPs, quantum size effect in semiconductor quantum dots, and super magnetism behavior in materials with magnetic properties. Interestingly, cutting down the dimensions of NPs has a clear-cut effect on the behavior of materials at nanoscale. These physical properties of NPs are due to their greater aspect ratio (surface area to volume ratio), spatial confinement, greater surface energy, and reduced imperfections. NPs have various applications in biomedical, cosmetics, optics, electronics, space technology, energy, catalysis, food, and many more (Rosarin and Mirunalini 2011; Solomon and D'Souza 2011).

For the NP synthesis, two different strategies, namely, "top-down" and "bottom-up," are used. In the former case, large-sized materials are converted to smaller ones with the help of reducing agents, whereas in case of bottom-up approach, the atoms are assembled into larger molecular structures at nanometer scale (Ali et al. 2015). An overwhelming number of chemical and physical processes are popular that are taken into account for manufacturing of metallic NPs (like silver, gold, copper, magnetites, and semiconductor quantum dots) using these two approaches. These conventional synthesis procedures are inefficient in material and energy usage, laborious, and capital-intensive and produce toxic wastes as end products (Thakkar et al. 2010) and involve the employment of harmful chemical substances, intense reaction conditions, and electrochemical techniques. To overcome these limitations associated with the traditional methods and to increase the

biocompatibility of nanocompounds, the research in the current scenario is directed to look for the reliable, eco-friendly, and sustainable approaches for the development of NPs of desirable shape and sizes with excellent properties of dispersity and stability causing minimal or no harm to the living systems and environment (Chauhan et al. 2011). In a way to achieve the extensive scope of nanocompounds, the potential of microorganisms is realized for the synthesis of metal NPs. The microbe-mediated synthesis of nanomaterial has attracted the great scientific world across the world.

Biogenic NPs synthesized by using the living entities like bacteria, fungi, and actinomycetes are highly stable with better specificity and catalytic reactivity as they help to maintain a better contact between the enzyme and metal salt because of microbial carrier matrix. The microbes are widely abundant in natural ecosystems and can be cultured in a suitable growth media (Li et al. 2011). Apart from the advantages of biological synthesis, the exploitation of microbial species for the manufacturing of nanoscale particles also possesses some drawbacks of culture maintenance which are quite complex and take a long duration to grow. The microbe-mediated synthesis approaches do not provide control over size distribution and shape of nanostructures. Even the formed biogenic NPs are polydisperse in nature and production rate is so slow (Narayanan and Sakthivel 2010). Despite of these problems, there are still chances to gain insight into the bacterial synthesis of NPs through the process optimization of reaction parameters such as microbial strain selection, age and concentration of culture used, pH, temperature and time of incubation of the reaction mixture, and concentration and type of metal salts used (Punjabi et al. 2015). The establishment and implementation of these optimization processes will provide hope for the use of NPs synthesized by microbes on a large scale and for marketable applications.

The biosynthesis of metallic NPs takes place when the microorganisms capture ions of interest from their surrounding environment and convert the metal ions into the elemental form of metals through the involvement of various enzymes released by the cell activities. Microorganism can synthesize metal NPs through two mechanisms, i.e., metal bioreduction and biosorption. The first is bioreduction, in which metal ions are chemically reduced into more stable forms biologically in which the reduction of a metal ion is coupled with the oxidation of an enzyme. Microorganisms possessing tendency of metal bioreduction can colonize metal-contaminated surroundings (Deplanche et al. 2010). Biosorption is a phenomenon in which microbes are surrounded by toxic heavy metals in their surroundings leading to stress conditions. The metal ions bind to the cell wall; the released peptides or other cell wall components bind to these metal ions and form stable nano-complexes (Yong et al. 2002).

The synthesis of NPs mediated by microbes occurs mainly by two routes, e.g., intracellular and extracellular. NP formation through the use of microbes is an example of bottom-up approach, where the first step of reaction deals with the reduction/oxidation of substrates and then the development of colloidal formations (Moghaddam 2010). In the intracellular mechanism, metal ions cross the microbial cell membrane and are transported inside to form NPs in the presence of enzymes. The microbial enzymes act as reducing agents responsible for reduction of metal

salts into nano-forms. Reduced metal atoms nucleate with subsequent growth for the generation of nanostructures. The extracellular approach of NP synthesis involves entrapping of the metal ions on the microbial cell surface and reducing the metal ions in the presence of enzymes (Zhang et al. 2011). Due to the biocompatibility of these biosynthesized metal NPs, they generally find applicability in the medical fields. The metal NPs find a wide space for the disease diagnostics as well as for the treatment of certain diseases. These metal NPs possess great antibacterial potential which allows the utility of these nanostructures in the medical fields. Microbe-mediated synthesis of metal NPs is a promising approach which connects microbial biology with biotechnology and nanotechnology. There is a need to explore this area to search the environmental-friendly and cost-effective procedures for the development of nanomaterials to realize the full potential of metal NPs in the medical as well as other spheres of life.

2 Role of Bacteria in Nanocompound Formation

Recently scientists have started focussing on prokaryotes for synthesis of metallic NPs. Due to their ubiquitous nature, abundance in environment, and ability to tolerate diverse conditions, bacteria are a good choice for this purpose. Bacteria are fast growing, easy to manipulate, and inexpensive to cultivate. Bacteria are considered as potential bio-candidate for the synthesis of metallic NPs like gold (Au), silver (Ag), platinum (Pt), palladium (Pd), titanium (Ti), and so forth. Bacteria tend to synthesize inorganic materials at nanoscale dimensions by either intracellular or extracellular mechanisms. Most of the ions of metal salts can cause harm to bacteria, and hence the metal reduction by the microbes is a particular kind of defense mechanism generated to protect them from such toxicity. Microbes become resistant to hazardous heavy metals because of chemical detoxification as well as outward movement of energy-dependent ions from the cell through some membrane-bound proteins which act as ATPase or as chemiosmotic cation or proton anti-transporters. Variation in solubility plays a deterministic job for microbial resistance. The location of the reductive components present in the cell also affects the formation of microbe-mediated NP synthesis. When the enzymes present on the cell membrane act as reducing agents and are involved in metal ion reduction, then extracellular formation of metal NPs is quite obvious. NPs produced by extracellular pathway have applications in different spheres, i.e., optoelectronics, electronics, bio-imaging, and sensing technology, than intracellular accumulation.

2.1 Intracellular Biosynthesis

For intracellular synthesis of NPs using microbial culture, the bacterial/fungal culture is grown in a specific liquid growth media incubated on a shaker at a certain optimal temperature. After complete incubation, the culture flask is kept under

static conditions to allow the microbial biomass to settle down; after that the culture supernatant is discarded and the cells are washed with distilled water (Punjabi et al. 2015). This step of cell washing is repeated several times and the supernatant is discarded each time. The biomass separated by centrifugation is further exposed to aqueous solution of metals at different concentrations. The culture is then incubated on a shaker at a suitable temperature until a visual change in color is observed. A color change from pale yellow to brownish color indicates the synthesis of silver nanoparticles (AgNPs); pale yellow to pinkish or purple color represents the formation of gold nanoparticles (AuNPs).

2.2 Extracellular Biosynthesis

NPs are synthesized by extracellular biosynthesis mechanism where the test microbial strain is grown in an appropriate growth media and incubated under particular growth conditions like temperature and shaking. After incubation, the culture broth is centrifuged and the supernatant is used for synthesis of NPs. The culture supernatant is added to the solution of metal ions at an optimized concentration and then incubated for a period of 72 h. The visible color change in the reaction mixture is noticed after a particular time which confirms the biosynthesis of NPs in the solution (Punjabi et al. 2015).

2.3 Synthesis of Metal Nanocompounds

In the recent times, prokaryotes are in high demand for the biosynthesis of NPs. The metal NPs are formed from the reduction of bulk metal salts. In this section, we have tried to summarize various metallic NPs like silver, gold, copper, magnetic NPs, and quantum dots synthesized using different species of microbes like bacteria, fungi, and actinomycetes.

2.3.1 Synthesis of Silver NPs (AgNPs)

AgNPs are those prepared from different silver salts like silver chloride ($AgCl_2$), silver iodide (AgI_2), and silver nitrate ($AgNO_3$) having size range of 1–100 nm. The important methods taken into account for the formation of AgNPs include the chemical and physical methods. But the only disadvantage behind the use of these two approaches includes high cost of preparation. To make the cost-effective synthesis, biological approach using microbes has been supposed as an alternate. Many bacterial strains used for the synthesis of AgNPs include either extracellular or intracellular synthesis approaches. Intracellular synthesis involves the use of extra materials like surfactants or ultrasound treatment to release synthesized NPs. Extracellular synthesis is cheap, requires downstream processing, and is useful for large-scale production of AgNPs.

AgNPs have been synthesized by the microwave irradiation of culture supernatants of *Bacillus subtilis*. Synthesized AgNPs have size in the range of 5–50 nm. Microwave irradiation resulted in uniform heating and reduction of aggregation around AgNPs (Saifuddin et al. 2009). AgNPs have been synthesized intracellularly and extracellularly using *Bacillus* strain CS11. $AgNO_3$ solution has been added to the nutrient broth containing bacterial biomass and incubated for 72 h at room temperature in the presence of light. A visible color change of the medium from pale yellow to brown indicates the formation of AgNPs. The mechanism for the bioreduction of silver ion to AgNPs is still unclear, although it is considered that some enzymes like nitrate reductase secreted by the microbes are responsible for the reduction (Das et al. 2014). AgNPs can also be synthesized by adding the $AgNO_3$ solution to the supernatant of the bacterial cultures of *Bacillus subtilis*, *Lactobacillus acidophilus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, *Staphylococcus aureus*, etc. The extracellular metabolites excreted by the cultures reduce the silver ions into AgNPs in the presence of light (Shahverdi et al. 2007). *E. coli* was also used for the extracellular synthesis of 40–60 nm-sized AgNPs. The effect of growth media and incubation period was examined on the formation of AgNPs and observed that luria broth (LB) medium and stationary phase have the maximum potential for the synthesis of AgNPs (Natarajan et al. 2010).

The use of fungal species has also been reported for the formation of AgNPs. The fungus-mediated formation of AgNPs is based on the mechanism that the fungal cells trap Ag ions on its surface followed by the reduction with the help of released enzymes. The fungal species *Aspergillus terreus* has been reported earlier leading to formation of AgNPs by extracellular NADH-dependent reductase enzyme (Li et al. 2012). Extracellular biosynthesis of AgNPs by the use of *Fusarium oxysporum* is based on the reduction of metal ions by nitrate-dependent reductase enzyme and shuttle quinone process (Duran et al. 2005). Fungi produce large amounts of AgNPs as compared to bacteria due to the secretion of a large amount of proteins by a fungus which is responsible for formation of AgNPs. The synthesis of AgNPs by other bacteria and their size range are tabulated in Table 1.

2.3.2 Synthesis of Gold NPs (AuNPs)

AuNPs are the particles ranging in dimensions of 3–150 nm which also known as colloidal gold and possess many distinctive properties which make them "star" among other NPs. AuNPs have a tendency to change the color of colloidal solutions depending upon their sizes. The important properties of AuNPs are high surface to volume ratio; optical, electrochemical, and catalytic properties; low toxicity; easy to prepare; easily dispersed in liquids; ease of surface modification; etc. Many bacterial strains have been used for the intracellular and extracellular synthesis of AuNPs. The use of microbes for this function is found to be easy, economical, and eco-friendly. A brief description about the microbial synthesis methods and few related examples for the preparation of AuNPs are mentioned.

Table 1 Size, range, and location of synthesis of different types of metallic nanocompounds by the use of different kinds of microbial species

Type of microbes	Synthesis location	Type of NPs synthesized	Size and shape of NPs	References
Pseudomonas antarctica	Extracellular	AgNPs	Spherical shaped, 6–13 nm	Shivaji et al. (2011)
Bacillus CS11	Extracellular	AgNPs	Spherical, 42–92 nm	Das et al. (2014)
Idiomarina sp. PR58–8	Intracellular	AgNPs	Spherical, 26 nm	Seshadri et al. (2012)
Pseudomonas meridian	Extracellular	AgNPs	Spherical shaped, 6–13 nm	Shivaji et al. (2011)
Humicola sp.	Extracellular	AgNPs	Spherical, 5–25 nm	Syed et al. (2013)
Pleurotus ostreatus	Extracellular	AgNPs	Grain shaped, 8–50 nm	Devika et al. (2012)
Aspergillus niger	Extracellular and Intracellular	AgNPs	Spherical shapes, 43–63 nm	Vanaja et al. (2015)
Proteus mirabilis PTCC 1710	Extracellular and Intracellular	AgNPs	Spherical, 10–20 nm	Samadi et al. (2009)
Penicillium chrysogenum	Intracellular	AuNPs	Spherical, triangle, and rod shaped, 5–100 nm	Sheikhloo and Salouti (2011)
Aspergillus sydowii	Extracellular	AuNPs	Spherical, 8.7–15.6 nm	Vala (2014)
Pseudomonas fluorescens	Extracellular	AuNPs	Spherical, 50–70 nm	Radhika Rajshree and Suman (2012)
Aspergillus niger	Extracellular	AuNPs	Spherical, 12.79 ± 5.61 nm	Bhambure et al. (2009)
Fusarium oxysporum	Extracellular	AuNPs	Shape not defined, 22 nm	Thakker et al. (2013)
Aspergillus fumigatus	Intracellular	AuNPs	Spherical, irregularly shaped with indefinite morphology, 85–210 nm	Bathrinarayanan et al. (2013)
Pseudomonas fluorescens	Extracellular	CuNPs	Spherical and hexagonal shaped, 49 nm	Shantkriti and Rani (2014)
Salmonella typhimurium	Extracellular	CuNPs	Spherical, 49 nm	Ghorbani et al. (2015)
Pseudomonas stutzeri	Extracellular	CuNPs	Spherical, 8–15 nm	Varshney et al. (2010)
Magnetospirillum magnetotacticum	Intracellular	Iron oxide NPs	Cuboctahedral, 50 nm	Lee et al. (2004)
Geobacter metallireducens	Extracellular	Iron oxide NPs	Tabular shaped with 20–200 nm length and 20–70 nm width	Vali et al. (2004)
E. coli	Extracellular	CdTe	Spherical, 2–3 nm	Bao et al. (2010a, b)

(continued)

Table 1 (continued)

Type of microbes	Synthesis location	Type of NPs synthesized	Size and shape of NPs	References
Phanerochaete chrysosporium	Extracellular	CdS	Grain shaped, 2.56 nm	Chen et al. (2014)
Fusarium oxysporum	Extracellular	CdTe	Polydisperse spherical, 15–20 nm	Syed and Ahmed (2013)
Brevibacterium casei	Intracellular	CdS	Polydisperse spherical, 10–30 nm	Pandian et al. (2011)
Aspergillus terreus	Extracellular	PbSe	Polydisperse spherical, 20–50 nm	Jacob et al. (2014)

The biosynthesis of AuNPs has been reported from *Pseudomonas aeruginosa* and *Rhodopseudomonas capsulata*, whereby the cell-free supernatant of these two strains is mixed with hydrogen tetrachloroaurate, indicating the color change of solution to purple or red wine, further confirming the formation of AuNPs. The pH of the solution is the deciding factor for the shape and size of NPs. At pH 4 and 7, nanoplates and spherical NPs of size range 10–20 nm are formed, respectively (Singh and Kundu 2014). Cubic AuNPs are formed when a filamentous cyanobacterium, *Plectonema boryanum* UTEX 485, reacts with aqueous Au $(S_2O_3)_2^{3-}$ solution at 25–100 °C for 1 month, and octahedral AuNPs are formed after reacting $AuCl_4^-$ at 200 °C for 1 day (Lengke et al. 2006). Different fungal strains are also known for both extracellular and intracellular synthesis of metallic AuNPs. *Phanerochaete chrysosporium* (fungal mycelium) is treated with $HAuCl_4$ under ambient conditions to form AuNPs within 90 min by the protein secreted by fungus itself. The extracellular and intracellular production of AuNPs is due to the secretion of enzymes lacase and ligninase by fungus, respectively (Sanghi et al. 2011). *Verticillium* species of fungus has also led to the formation of AuNPs by the reduction of aqueous $AuCl_4^-$ ions (Mukherjee et al. 2001). The enzymes present within the cell wall of fungi are known to reduce the ions. A species-specific NADH-dependent reductase, released by *Fusarium oxysporum*, has also been used for reducing $AuCl_4^-$ ion into AuNPs (Mukherjee et al. 2002).

Thermomonospora, an extremophilic actinomycete, also reduces Au ions to AuNPs extracellularly. The harvested biomass is added to solution of chloroauric acid and kept in dark for the synthesis of monodisperse AuNPs, whereby enzymatic processes play a deterministic job in the reduction of metal ions as well as AuNP stabilization. The proteins secreted by actinomycete biomass act as capping agents for the stabilization of AuNPs (Sastry et al. 2003). The biomass of other actinomycetes, *Streptomyces viridogens*, has also been added to chloroauric acid solution, and the color of biomass changes to pink within 24 h of time indicating the formation of AuNPs within the cells. These synthesized AuNPs possess antibacterial activity against *S. aureus* and *E. coli* (Balagurunathan et al. 2011).

2.3.3 Synthesis of Copper NPs (CuNPs)

CuNP synthesis is a great challenge as Cu at nanometer scale is not so stable and easily oxidized to get converted into copper oxides (CuO). Therefore, it is of great need to further stabilize CuNPs after their synthesis so as to use them in various applications. Copper as metal or CuO at nanoscale possess unique properties as it is an essential part of living beings that plays a beneficial role in biomedical applications. The research on CuNPs has become a focal point due to their unique properties and low cost of preparation and utility in a wide array of applications. CuNPs hold good thermal and electrical conductivity, as well as optical properties.

A microbial approach has been used for the synthesis of CuNPs by incubating copper sulfate solution with the cell pellet and cell-free supernatant of *Pseudomonas fluorescens*. Spherical- and hexagonal-shaped NPs of 49 nm were formed (Shantkriti and Rani 2014). The supernatant of another bacterial culture *S. typhimurium* was incubated with aqueous solution of copper nitrate to form CuNPs of size 49 nm (Ghorbani et al. 2015). Fungi, such as *Penicillium* sp. and *F. oxysporum* strains, have also been reported to biosynthesize CuO and Cu_2S NPs. A fungus known as *Stereum hirsutum* has also been used for the synthesis of Cu and CuO NPs (Cuevas et al. 2015). The synthesis of Cu or CuO NPs can lead to different SPR which is formed due to the strong coupling between incident electromagnetic radiation and surface plasmon in metal NPs.

2.3.4 Synthesis of Magnetic NPs (MNPs)

MNPs are of great potential in the present era as they possess magnetic behavior with utility in diverse fields. The researchers pay more attention to the synthesis methods of MNPs so as to form uniform-sized MNPs because the properties of MNPs are size dependent. The three major functional parts of an MNP carrier include a magnetic core, a surface coat, and a functional outer coating. The inner magnetic core consists of a supramagnetic molecule (Fe, Ni, Co, etc.) which depends upon its application. The surface coat is used to provide steric repulsions, to increase the stability and to restrain agglomeration of the particles. The functionalized outer coating may attach any ligand or the biologically active entity (Kumari et al. 2014).

Magnetotactic bacteria (MTB) produce magnetic nanocrystals or MNPs which are enveloped by certain biomembranes called magnetosomes (Alphandery et al. 2008). They biomineralize membrane-bound magnetic nanocrystals of the iron oxide (Fe_3O_4) or iron sulfide (FeS_4) (Bazylinski and Frankel 2004). Inside the bacterial membrane, the magnetosomes are organized in a chain-like manner which offer strong magnetic dipole for bacteria to help move along the earth's magnetic field direction (Alphandery et al. 2008). MTBs are found in marine and freshwater sources. Freshwater milieus were found to contain various morphological types of MTB, including rod-shaped, comma (vibrio), coccoid, and helicoidal

forms. *Alphaproteobacteria* and *Magnetobacterium bavaricum* are some of the MTBs found in freshwater environments. Both $Fe_3O_4^-$ and $Fe_3S_4^-$ producer MTBs have been found in marine environments.

The strains of *Magnetospirillum magneticum* produce either Fe_3O_4 magnetic NPs in chains or Fe_3S_4 (greigite), while some other strains produce both types of NPs (Roh et al. 2001). *Fusarium oxysporum* and *Verticillium* species are able to form iron oxides mainly Fe_3O_4 by hydrolyzing the ion precursors extracellularly (Bharde et al. 2006). Magnetosomes can have square-like, rectangular, hexagonal, or bullet-shaped projections.

2.3.5 Synthesis of Quantum Dots (QDs)

Semiconductor nanocrystals are the type of inorganic NPs discovered in the early 1980s ranging in size between 1 and 10 nm. These represent a state of matter in the transition regime between the molecules and the bulk solid. A layer of organic ligands at the surface of semiconductor NPs stabilizes them in the colloidal form. The ligand consists of two parts: a polar head group that possesses the affinity for the attachment to the surface of semiconductor nanocrystals and, a second part, a tail, which helps in the solubilization of semiconductor nanocrystals in the organic media. The properties of semiconductor nanocrystals arise due to the spatial arrangements of atoms in the crystalline lattice. The optical and electronic properties of semiconductor nanocrystals can be varied with the reduction in the size of nanocrystals, and the phenomena can be described by the term "size quantization effect." The quantum dots (QDs) are the small-sized semiconductor nanocrystals to be fit in the quantum confinement region.

A facile and biocompatible route has been developed to synthesize CdSe QDs using *Escherichia coli* cells as a matrix. The QDs extracted from such cells showed a surface protein layer which acted to improve the biocompatibility of QDs (Yan et al. 2014). Yeast cells have been reported to synthesize biocompatible CdTe QDs of size 2–3.6 nm with easily tunable florescence emission capacity. An extracellular growth pathway is known to be responsible for the formation of the protein-capped CdTe QDs (Bao et al. 2010a). The fungus *Fusarium oxysporum* has been reported to synthesize CdTe QDs extracellularly. There is no need to add stabilizing agents as formed QDs are already capped by a natural protein (Syed and Ahmad 2013).

3 Factors Affecting NP Synthesis by Microbes

The size and shape of NPs are altered by the variation in certain physical and chemical properties of NPs. Various factors affecting the synthesis of NPs by the microbes include the age and concentration of cell culture used, nature and concentration of metal ion used, pH, temperature of reaction mixture, and incubation time which plays a great task in the microbe-mediated synthesis of NPs. The role of each factor and how it affects the synthesis of NPs are described below.

3.1 Age and Concentration of the Microbial Cell Culture

The age and concentration of the microbial culture used for the synthesis of metallic NPs have deterministic effect on the size and shape of NPs as the metabolic activities of the cells vary with the age. Age of culture has pronounced effect on NP synthesis (Maliszewska and Puzio 2009). With the increase in concentration of biomass used, there is an increase in the production of NPs. It is speculated that the enzymes present in the organisms also known as biocatalysts are responsible for biological synthesis of NPs. These biocatalysts are used in the forms of purified enzymes, whole cells, and crude enzymes. In respect to find the optimum age of cultured microbial cells for NP formation, the cultured cells are harvested at different phases of growth (lag, early stationary, late stationary). For example, synthesis of AuNPs using cell-free supernatant of *Geotrichum candidum* showed that the cell mass harvested at 48 h of growth produced maximum amount of AuNPs. The reason behind may be due to the emergence of maximum amount of reducing agents at 48 h growth which resulted in higher reduction of metal salts into NPs (Mittal et al. 2013).

3.2 Concentration of Metal Salt/Substrate Used

In biotransformations, one of the important factors is to find the maximum concentration of substrate which could be converted into final end product which makes the reaction more cost-effective and efficient. Concentration of initial substrate/metal ion used has a predominant effect on the shape and size of synthesized NPs. Increasing the concentration of silver nitrate solution from 1 mM to 5 mM in the reaction mixture is known to increase the particle size. Even the particle agglomeration is known to occur when high concentrations of metal salts (10 mM) are used in the synthesis reaction and also the production of AgNPs decreased. It is also reported that the use of higher concentration of $AgNO_3$ has certain toxic effects on *F. oxysporum* biomass (the biocatalyst). For the biosynthesis of AgNPs, the optimum concentration of silver nitrate should be around 5 mM (Korbekandi et al. 2013). Similar effects of metal salt concentration are noticed on the synthesis of AuNPs by the use of *Penicillium crustosum* where the increase in concentration of $AuCl_4$ (mM) causes a decrease in the average diameter of particles but size begin to increase at even higher concentration of salt used (Barabadi et al. 2014). This is explained based on the fact that increase in the concentration of metal salt used for reduction into nanoscale-sized particles allows the growth of NPs at a faster rate. Moreover, particles in higher salt concentration may have a tendency to aggregate and produce bigger particles.

3.3 pH

pH of the reaction mixture is a critical factor which influences the size and shape of NPs at a large extent. AgNPs of different size and shape can be obtained by controlling the environment of NP synthesis (Correa-Llanten et al. 2013). Increasing pH resulted in the formation of AgNPs of size range 10–20 nm, while decreasing the pH to 4 resulted in the formation of silver nanoplates (Correa-Llanten et al. 2013). In another study, acidic pH resulted in the formation of 45 nm-sized AgNPs, whereas at pH 10, AgNPs obtained were of just 15 nm. The pH was found to be a vital factor also affecting the synthesis of AuNPs in microbial cultures. Alterations in pH during contact to Au ions have a direct effect on the shape, size, and number of NPs formed per cell. AuNPs synthesized in *V. luteoalbum* after exposure to $HAuCl_4$ for 24 h at pH values of 3, 5, 7, and 9 were examined in a study by Gericke and Pinches (2006). AuNPs formed at pH 3 were comparatively uniform in size where majority of NPs were of spherical shape having a diameter less than 10 nm. At pH 5, the synthesized AuNPs were small spherical particles, similar to those NPs formed in the reaction mixture at pH 3. Apart from this, a great quantity of large-sized NPs of few more variable shapes, e.g., triangles, spheres, hexagons, and rods, are also synthesized at this pH. The shapes of formed AuNPs at pH 7 were similar to particles observed at pH 9 which included small spherical as well as bigger particles of irregular, undefined shapes. Another study where AuNPs have been synthesized by whole cell supernatants of *Geotrichum candidum* demonstrated that the synthesis of AuNPs is optimum at pH 7, whereas at pH above and below 7, particle aggregation is observed (Mittal et al. 2013). The synthesis of metal NPs of variable shapes by the use of reaction media at different pH values signifies that the variation in this factor tends to control the particle morphology during optimization of a synthesis process.

3.4 Temperature

The environment of NP synthesis such as temperature, oxygenation, and incubation can be easily controlled and manipulated. The size of AgNPs is also affected by the reaction temperature. Increase in temperature leads to the formation of smaller-sized AgNPs. The formation of many seed crystals is the reason behind the shape-controlled synthesis of AgNPs by controlling certain environmental factors. Lower (acidic) pH and lower temperatures of the reaction mixture lead to lesser nucleation for the formation of AgNPs where new Ag atoms get deposit to build large-sized NPs. But with an increase in pH as well as temperature, nucleation rate increases due to a great abundance of hydroxyl ions and raised temperature. The increase in

formation of AgNPs is followed by an increase in the kinetics of the deposition of the silver atoms (Correa-Llanten et al. 2013). The whole cells of *Geotrichum candidum* exposed to tetrachloroauric acid at a concentration of 1 mM was added to resuspended whole cells, and then culture flasks were incubated in a shaker at different temperatures in the range of 15–40 °C to form AuNPs. An increase in reaction rate up to a certain value was observed with increase in reaction temperature which further started decreasing. A temperature of 35 °C was found to be optimum for NPs synthesis, beyond which the absorption at 520 nm decreased. This may be due to instability of reductive compounds (protein/peptide) at higher temperatures (Mittal et al. 2013).

3.5 Incubation Time

The time taken to complete NP synthesis is an important parameter from an industrial point of view. Bai et al. (2006, 2009) have noticed that the size of NPs increases due to the increase in incubation time during the formation of ZnS and CdS NPs by using the bacterial species, *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides*. During the synthesis of CuNPs using cell pellet or cell-free supernatant of *Pseudomonas fluorescens* after incubation with $CuSO_4$ solution, effect of incubation time was studied. It was noticed that optical density (absorbance) at 610 nm increased gradually up to 90 min and then decreased which revealed the formation of CuO NPs. Further, with an increase in time, size reduction takes place (Shantkriti and Rani 2014).

4 Mechanism of Nanocompound Formation by Microbes

The mechanism of synthesis of NPs mediated by microbes is different for synthesis by different organisms. A general mechanism of formation of NPs is that, firstly the metal ions got entrapped on the surface or enter inside the bacterial cells, and then the metal ions are reduced to NPs by the release of certain factors by microbes like enzymes or proteins. This part mainly focuses on the possible action of mechanism for synthesis of typical types of NPs (AgNPs, AuNPs, CuNPs, MNPs, and QDs) by microbes. A diagrammatic representation of mechanistic action of NPs is illustrated in Fig. 1.

4.1 Mechanism for the Synthesis of AgNPs by Bacteria

All the organisms do not have capability to synthesize AgNPs. As reported earlier, those organisms which possess the "silver resistance machinery" have the

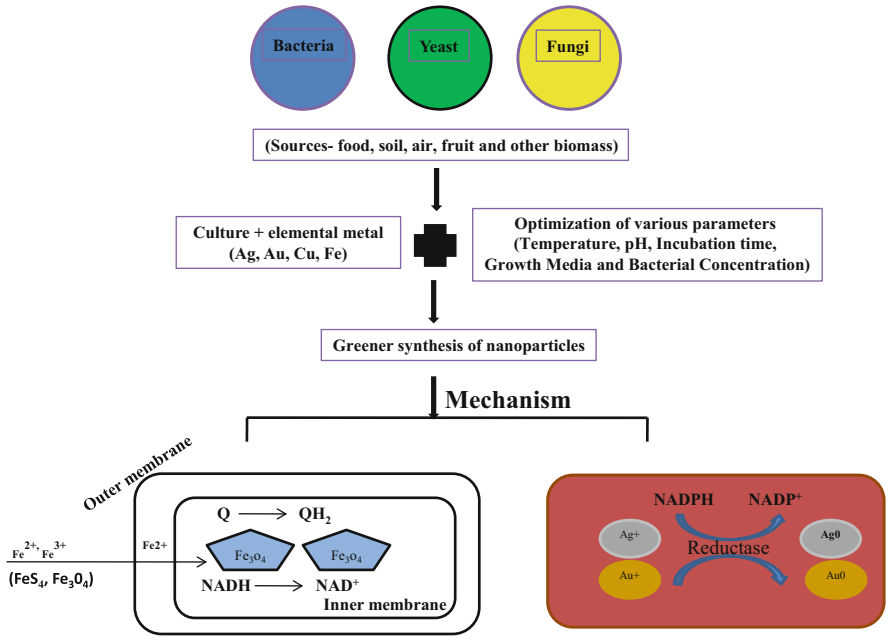

Fig. 1 A brief representation of mechanistic action of microbe-mediated green synthesis of nanocompounds

capability of AgNPs synthesis while the mechanism of resistance differs with the organisms. Bacterial cell extracts may serve as both the reducing and capping agents for the formation of AgNPs. The reduction of silver ions by various components like enzymes/proteins, amino acids, and polysaccharides present in bacterial cellular extracts is found to be eco-friendly but chemically complicated. The presence of enzyme "nitrate reductase" is responsible for the synthesis of AgNPs. It is postulated that nitrate reductase is the causal agent behind the synthesis of AgNPs in *B. licheniformis*. Nitrate reductase is induced by nitrate ions which reduce silver ions to metallic silver. NADH- and NADH-dependent nitrate reductase enzymes play a vital role for synthesizing AgNPs. *B. licheniformis* secretes the cofactor NADH- and NADH-dependent enzymes, especially nitrate reductase, which might be responsible for the reduction of Ag^{+} to Ag^{0} and the subsequent formation of AgNPs (Husseiny et al. 2007). Moreover, in alkaline reactions, the synthesis of AgNPs is faster as compared to the reaction in acidic conditions. Synthesis of AgNPs increases as the pH of reaction mixture is raised toward alkaline scale and reaches to maximum at pH 10. If the reaction pH is increased beyond 10, then the AgNP synthesis starts declining. The proteins containing thiol groups (−SH) bind with silver forming a –S–Ag bond, responsible for the synthesis which provides an idea about the conversion of Ag^{+} to Ag^{0}. In addition, the alkaline

ion (OH) is required for the metal ion reduction. Under normal conditions, it requires generally 4 days for the complete formation of silver ions, whereas less than an hour is taken when the pH is made alkaline. Alkalinity increases the capacity of the enzymes which play a role for AgNP synthesis.

4.2 Mechanism of Synthesis of AuNPs by Microbes

A wide variety of microbes are known for the formation of AuNPs. A general mechanism behind the microbe-mediated formation is the reduction of gold (Au^{+3}) ions to Ag^0 to form AuNPs. It has been speculated that the enzymes secreted by various microbes help in reduction of metal ions, causing nucleation and growth of NPs. The synthetic route of AuNPs involves the reduction of gold salts by a few reducing agents that happen either in the extracellular or intracellular environment. In a study using filamentous cyanobacterium, *Plectonema boryanum* UTEX 485 for the synthesis of AuNPs showed that in abiotic experiments, the solutions of gold sulfide were found to be stable for about 1 month at 25 °C, whereas AuNPs of size ~25 nm having cuboctahedral shape were precipitated at 60–200 °C. Similar is the case observed with the solutions of $AuCl_4$ which were found to be stable for 1 month at 25–60 °C, but at higher temperatures of 100–200 °C, AuNPs of irregular shape (~25 nm) were formed. Increase in temperature from 25 °C to 60–200 °C leads to an increase in precipitation of gold. When the bacterial cells were exposed to high concentrations of gold salt aqueous solution of gold chloride, they lead to the production or release of membrane vesicles for the protection of cells. The interaction of vesicle components like proteins, lipopolysaccharides, and phospholipids with the gold salts leads to the precipitation of gold on the vesicular surfaces or in solutions (Lengke et al. 2006).

Extracellular synthesis of AuNP formation is commonly reported by fungi *F. oxysporum* which releases protein-reducing agents into the solution. When Au^{3+} ions from gold chloride salt are trapped and reduced by protein reductase in the cell wall, these protein molecules bind to AuNPs through the linkage between the amine groups in amino acid lysine (Mukherjee et al. 2002). AuNP formation by intracellular approach and diffusion of Au^{3+} ions from the gold salt solution take place through the cell membrane and are then reduced by cystolic redox mediators. The different types of functional groups present on the cell surface like carboxyl, amine, phosphate, etc. provide binding sites for Au(III) for mineralization of Au. Negatively charged gold ions bind on the positively charged mycelia of *R. oryzae* through electrostatic interaction with phosphoproteins (Das et al. 2012). However, it is still unexplored which need extensive research whether the diffusion of Au^{3+} ions through the membrane occurs via active bioaccumulation or passive biosorption. The biosorption might be caused by toxicity of Au^{3+} ions, which can increase the porosity of the microbial cell membranes.

4.3 Mechanistic Action of Microbial Synthesis of CuNPs

Different species of microorganisms have different mechanistic action for the synthesis of CuNPs. The bacteria take target metal ions from the surroundings on their cell surface or inside the membrane. The captured metal ions are reduced to NPs in the presence of reductant enzymes released by the cell activities. The interaction between the positively charged metal ions and negatively charged carboxylate functional group of the enzymes located in the cell wall causes the reduction of the metal ions (Li et al. 2011). Another study explains the formation process of CuNP nanoparticle in *Serratia* sp. The bacterial cells in stationary phase face an oxidative stress and osmotic stress because of the exhaustion of energy source and increase of metabolic wastes. When the cells are exposed to high concentration of metal salts, the stress is increased even more. The metal salt is internalized into the cell membrane, and Cu^{2+} ions get reduced by particular biomolecules to metallic Cu, which is comparatively less toxic which further lead to the production of CuNPs (Hasan et al. 2007).

4.4 Mechanism of MNPs

The molecular mechanism of bacterial magnetic particle (BacMP) biomineralization is a multistep reaction (Arakaki et al. 2008). First, the cytoplasmic membrane invaginates, and then the vesicle is produced which acts as the starting material for the formation of the BacMP membrane. A specific GTPase helps in the priming of the invagination for vesicle formation of magnetotactic bacteria just alike other eukaryotic organisms. Along with cytoskeletal filaments, the vesicles organize themselves in a linear chain structure. The next step of biomineralization includes the accumulation of Fe^{3+} into the vesicles by the transmembrane iron transporters, transport proteins, and siderophores. Fe accumulation is further controlled by a redox system. Magnetite crystal nucleation is the last step regulated by various proteins related with the BacMP membrane for magnetite generation. These include the accumulation of supersaturating iron concentrations, maintenance of reductive conditions and the oxidation of iron to induce mineralization, or the partial reduction and dehydration of ferrihydrite to magnetite (Arakaki et al. 2008).

Another most feasible mechanism takes into consideration both passive and active mechanisms for the manufacturing of magnetites using *Shewanella oneidensis* (Perez-Gonzalez et al. 2010). Firstly, by active mechanism, the formation of Fe^{2+} occurs when bacterial species make use of ferrihydrite as a terminal electron acceptor, and the pH in the cell surrounding environment increases most likely due to the amino acid metabolism. Then, through a passive mechanism, the local accumulated concentration of Fe^{2+} and Fe^{3+} at the net negatively charged cell structures and cell wall provokes a rise of supersaturation of the system with respect to magnetite, causing the precipitation of magnetite to form NPs.

4.5 Mechanism of Synthesis of Quantum Dots

The precursor molecules like $CdCl_2$, Na_2TeO_3, mercaptosuccinic acid, and sodium borohydride are incubated with *E. coli* in LB medium. The uniform-sized CdTe QDs capped with proteins are formed with good crystallinity as a result of mechanism of extracellular growth which encompassed the nucleation reaction of metal ions with the protein molecule yeast secreted by bacteria and then followed by Ostwald ripening (Bao et al. 2010b). It could be assumed that *E. coli* cells exposed to extreme environmental conditions of toxic heavy metal ions require starting of a specific kind of defense mechanisms to protect them from the unfavorable stress developed by metal ion. To overcome the metal stress, the bacterial cells generate more metal-binding proteins which promote the synthesis of QDs by microbes. More research needs to be carried out to confirm the mechanism proposed above. In another report, it was described that *S. cerevisiae* yeast cells were first incubated with Na_2SeO_3 in a suitable growth media. Then the harvested seleniumized yeast cells were further cultured with $CdCl_2$-containing media which resulted in in situ synthesis of CdSe QD. Glucan content increases in the cell walls of yeast cells which result in enhanced mechanical strength (Luo et al. 2014).

5 Multi-scale Characterization of Nanocompounds

The characterization of biosynthesized nanocompounds is very essential as the properties like size, shape, surface functional groups, thermal conductivity, and surface charge of NPs determine their behavior in the body systems which are required for their medical applicability. A large number of high-throughput instruments are available which are used for the characterization of NPs of microbial-mediated synthesis. As the size, shape, and other properties of NPs vary with the variation in reaction parameters, concentration of precursor molecule used, type of microbial culture used, and the synthesis route, it is a necessary task to determine these properties before the use of NPs for different purposes. The characterization gives a primary clue about the behavior of NPs in the living systems. The following characterization techniques are used to determine the essential characteristics of NPs.

5.1 Scanning Electron Microscope (SEM)

SEM is used to estimate the surface topography of NPs. An electron beam of high energy strikes the sample surface and then interacts with the atoms at or near the sample surface. The signals produced by SEM include secondary electrons,

backscattered electrons, and characteristic X-rays which provide details about the topography and elemental composition of the sample (Joshi et al. 2008). SEM can be used to observe only the external morphology of the sample. The sample preparation of NPs is comparatively simple to analyze the sample using SEM. Vanmathi Selvi and Sivakumar (2012) used SEM to estimate the size of AgNPs biosynthesized by *F. oxysporum*. SEM micrographs showed the spherical-shaped AgNPs of 20–25 nm.

5.2 Transmission Electron Microscope (TEM)

A beam of electron interacts with the interior of the sample mainly through diffraction. The lenses, deflection coils, and stigmators help in the formation of image and then project it on the screen. The projection chamber having different types of electron detectors help to record the sample images. A focused electron beam when passes through an extremely small thin sample provides an insight into size, shape, composition, NP localization, crystallinity, and other characteristics (Punjabi et al. 2015). TEM provides the internal structure of the sample at a very high magnification power. The only limitation of TEM is laborious procedure of sample preparation and expensive. TEM was used to characterize the intracellular localization and size of AuNPs synthesized by *Geobacillus* sp. The quasi-hexagonal-shaped AuNPs of size 5–50 nm were observed (Correa-Llanten et al. 2013).

5.3 Atomic Force Microscope (AFM)

The sample surface is scanned using a probe and the oscillation amplitude is used to measure the surface characteristics of the sample (Joshi et al. 2008). AFM provides the three dimensional structures of the NPs. It provides higher resolution as compared to SEM. Instead of using high-energy electron beam, a laser light is used to measure the deflection of cantilever probe. In a study, AFM was used to study the influence of *Microbacterium hominis* and *Bacillus licheniformis* extracellular polymers on AgNPs and iron oxide NPs which revealed that the formed AgNPs were of size less than 100 nm, whereas iron oxide NPs were of 100–200 nm (Gholampoor et al. 2015).

5.4 Dynamic Light Scattering (DLS)

DLS is a technique which is used for determining the size of particles in the submicron region. The principle is based on the Brownian motion of particles

which relates it to their sizes in the suspended form. Brownian motion is based on the random movement of particles as the particles strike with solvent molecules in the surrounding atmosphere. DLS measures the hydrodynamic diameter of particles when they are surrounded by solvent molecules. The size, size distribution, and surface charge of NPs can be estimated by DLS (Punjabi et al. 2015).

5.5 UV-Vis Spectroscopy

Metal NPs scatter optical light in the UV-Vis region of the spectra because they possess surface plasmon resonance (SPR). NPs show a characteristic SPR peak in UV absorption spectra. The peak intensity, wavelength at which NPs show absorbance, and spectral bandwidth particularly depend on size, shape, aggregation behavior, and material composition of NPs (Joshi et al. 2008). UV-Vis spectroscopy provides a primary confirmation about the formation of NPs.

5.6 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR provides knowledge about the surface functional groups present on NPs. The knowledge provided helps to determine the presence and interaction of reducing agents and the capping agents which are responsible for synthesis and stability of NPs. The reports are available where researchers have used FTIR to study the mechanism of action of microbial synthesis of metal NPs which revealed the information regarding the protein or polysaccharide-reducing agents responsible for the biosynthesis of NPs.

6 Medical Applications of Nanocompounds

Out of plethora of applications, NPs are versatile agents for utility in biomedical prospects such as diagnostics, cancer targeting, disease treatment, antimicrobial activity, tissue engineering, and many more. Additionally, the nanotechnology-based approaches can also be beneficial in diagnostic as well as therapy simultaneously. The term "theranostics" has been explored for the unique properties of NPs possessing better penetration of therapeutic moieties and tracking within the body so as to allow a more efficient therapy with a reduced risk in comparison to conventional therapies.

6.1 Antimicrobial Activity

The antimicrobial agents are those that kill or dampen the growth of microbes such as bacteria, fungus, algae, etc. The emergence of infectious diseases poses a great threat to human health mainly occurring due to antibiotic-resistant microorganisms. Due to the overuse of antibiotics, microorganisms become resistant to variously used antimicrobial agents. Nanotechnological advances are used to synthesize antimicrobial NPs with different shape and size to control the bacterial growth to a benchmark level. NPs are seeker of great attention in various fields but mainly medicines as the functionality of NPs is based on particle size (Thomas et al. 2014). The ability to easily manipulate or control the dimensions of NPs creates the opportunities for the development of new nanotechnology-based antimicrobial agents.

A variety of metallic NPs and metal oxides including Ag, Au, Cu, Ti, Zn, etc. are known to inhibit the growth of several species of bacteria, fungi, and viruses. The inhibition of bacterial growth is influenced by factors such as the size of NPs, concentration used, and their stability. The smaller is the size of NPs, the more is the concentration used in the media and then the more will be the bacterial inhibition. The size of bacterial cell lies in a micrometer range, whereas the pores of the bacterial cell membrane has a diameter in nanometer dimensions, so it would be a better choice to use NPs of size smaller than bacterial pores (Ashajyothi et al. 2014). The small-sized metal NPs will be able to cross the bacterial cell membrane and enter the cell to destroy the proteins, thus leading to hampering of bacterial growth. The schematic representation of mechanism of antimicrobial action of metal nanocompounds is presented in Fig. 2. Antibacterial activity of biosynthesized nanocompounds against a broad range of microbes is given in tabular form (Table 2).

6.2 Imaging for Diagnostics

6.2.1 Imaging for Disease Diagnostics

Metal NPs particularly Au and Ag have been found of wide applicability in diagnostics due to SPR effect and SERS properties (Kneipp et al. 2010). Such NPs have led to the development of nanoprobes used for imaging because of excellent features of size range ($\leq$100 nm), enhanced surface area, and different behavior from their bulk counterpart (Na et al. 2009). Kim et al. (2007) have developed polyethylene glycol (PEG)-coated AuNPs (30 nm) as a contrast agent for in vivo X-ray computed tomography (CT) imaging. Intravenous injection of these coated AuNPs into hepatoma-bearing rats showed a twofold high contrast between hepatoma and normal liver cells confirming the use of PEG-coated AuNPs as a CT contrast agent for hepatoma imaging. Earlier some problems of short

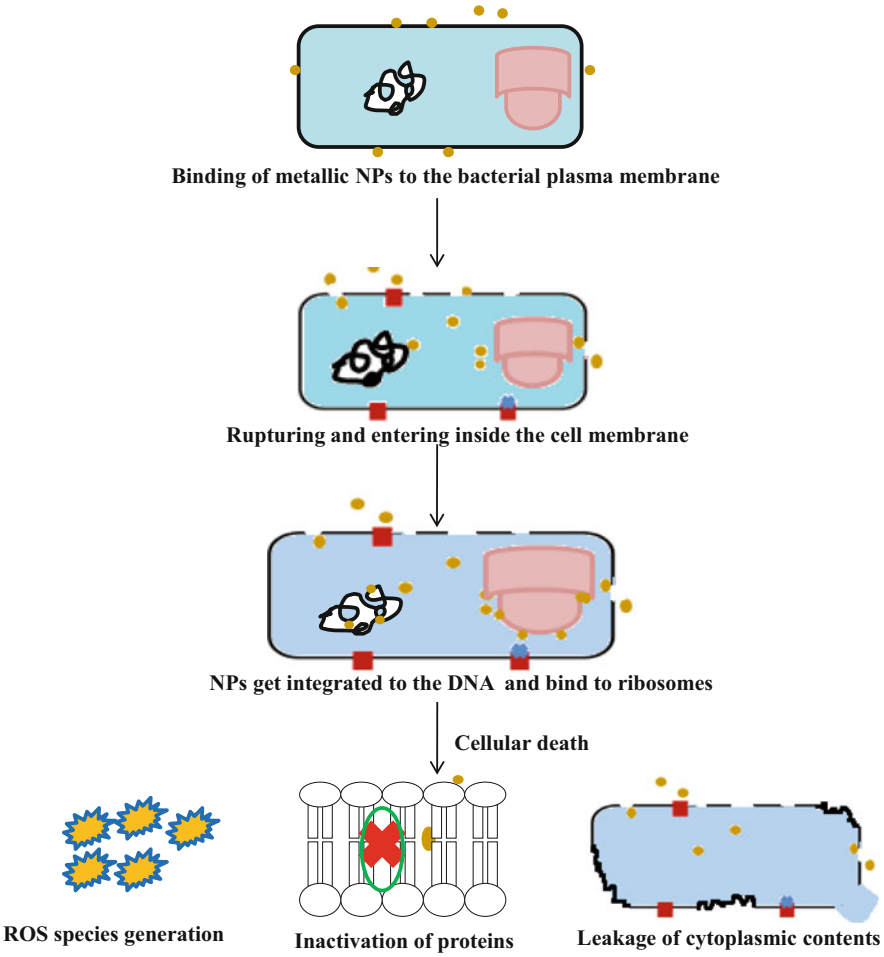

Fig. 2 Flow diagram representing the antimicrobial action of metallic nanoparticles

imaging time due to fast clearance by kidneys were faced with the use of iodine-based compounds in CT imaging. The use of AuNPs as contrast agent in CT imaging has overcome these limitations. Pan et al. (2012) have reported for the first time the use of 80–90 nm-sized self-assembled neodecanoate CuNPs that act as contrast metal NPs for the detection of sentinel lymph node in the near-infrared region using photo acoustic tomography (PAT). These NPs showed a higher sixfold increase in signal sensitivity when compared to blood which is a good absorber of light. The clinical applications of these CuNPs may serve as substitute of sentinel lymph node biopsy in the coming future generations.

MNPs have also been used as MRI contrast agents because of their magnetism, nanoscale size, low toxicity, and biocompatibility. Some of these NPs possess higher relaxivity, higher magnetization, and different types of magnetism based

Table 2 A tabulated form of various biosynthesized nanocompounds showing strong antimicrobial action against broad spectra of microbes

Microbe responsible for synthesis of NPs	Type of NPs synthesized	Antibacterial activity against microbes (zone of inhibition in mm)	Size and conc. Used	References
Fusarium semitectum	AgNPs	*K. pneumoniae* (15) and *P. aeruginosa* (16)	8–50 nm; 50 µl	Shelar and Chavan (2014)
Ochrobactrum anthropi		*S. typhi* (14), *Salmonella paratyphi* (15), *Vibrio cholera*(16), *S. aureus* (15)	38–85 nm; 40 µl	Thomas et al. (2014)
P. aeruginosa KUPSB12		*E. coli* (19), *Vibrio cholerae* MTCC 3904 (15), *Shigella flexneri* MTCC 1457 (16), *B. subtilis* MTCC 441 (17), *S. aureus* MTCC 3160 (13), and *M. luteus* MTCC 1538 (18)	50–85 nm; 25 µl	Paul and Sinha (2014)
E. coli		*B. subtilis* (18), *K. pneumoniae* (20), *E. coli* (15), *P. aeruginosa* (15), and *S. aureus* (13)	10–100 nm; 200 µl	Veeraapandian et al. (2012)
Gracilaria corticata	AuNPs	*E. coli* (24), *Enterobacter aerogenes* (21), *S. aureus* (19), and *Enterococcus faecalis* (14)	45–57 nm; —	Naveena and Prakash (2013)
Galaxaura elongata		*S. aureus* (13), *Escherichia coli* (17), *K. pneumonia* (16), and *P. aeruginosa* (13)	3–77 nm; —	Abdel-Raoul et al. (2013)
Enterococcus faecalis	CuNPs	*E. coli* (25), *K. pneumoniae* (18), methicillin-resistant *S. aureus* (25)	20–90 nm; 60 µl	Ashajyothi et al. (2014)

on their core-shell structure and even functional aspects. Zeng et al. 2012 have demonstrated the use of ultrasmall, water-soluble iron oxide NPs (Fe_3O_4, $ZnFe_2O_4$ and $NiFe_2O_4$ NPs) synthesized in aqueous solutions, as T1-weighted contrast agents for MRI. These ultrasmall iron oxide NPs possessed strong T1-weighted relaxation and weak T2 relaxation properties. In another approach, the effect of size and composition of iron oxide NPs to tailor MRI and synthesized iron oxide NPs of spherical, cubical, and octopod shape by thermal decomposition method has been described. The results documented that at ultralow concentrations, the spherical iron oxide NPs displayed high in vitro and in vivo MRI functionality, whereas cubical-shaped NPs showed positive contrast for T1-weighted images and negative contrast for T2-weighted images confirming the use of these iron oxide NPs as high-contrast agents in MRI. Supra-paramagnetic iron oxide nanoparticles (SPION) of spherical shape display T2 negative contrast (Walter et al. 2014).

6.2.2 Imaging for Cancer Detection

Cancer or tumor detection at an early stage may decrease the chances of tumor progression and helps to get successful treatment so as to provide quality life to patients. In order to detect or diagnose the tumor at initial stages, it is essential to detect certain tumor markers, macrophages, or circulating tumor cells to have information of cancer screening and diagnosis. During early phases of disease, determination of cancer tissue-specific biomarkers at ultralow levels requires numerous strategies which take into consideration the usage of various bionanocomposites having high degree of specific and sensitive tags of detection for tracing out signals (Du et al. 2010). The nanotechnological approaches are appropriate to design certain functionalized nanoscale materials which play a real role in cancer diagnosis as compared to conventional chemotherapeutic agents.

Metallic NPs have been proven as one of the astonishing nanomaterials for cancer detection because of their size, shape, composition, easy preparation, light absorbance and scattering potential, and size-dependent optical properties. Metal NPs, particularly Ag and Au, have the capacity to scatter light in the wavelength of visible and near-infrared region upon the excitation of their SPR. The scattering light intensity is highly sensitive to the size and aggregation of NPs. An attempt has already been done to use AgNP-based SERS to analyze and screen human blood plasma to develop a simple and label-free blood test for esophageal cancer detection which proved a great potential of these AgNPs for improving cancer detection and screening (Du et al. 2010). A spherical-shaped AuNPs have been reported as optical probes for the detection of prostate cancer biomarker using immunoassay where AuNPs of diameter 37 nm have been conjugated with antiprostate-specific antibodies for detection purpose (Liu et al. 2008). In another report, AuNPs (60–80 nm) biosynthesized by *C. albicans* have been conjugated with the specific antibodies against the surface of liver cancer cells. The developed bioconjugated AuNPs were found to bind with the antigens present on the surface of cancer cells in a specific manner (Chauhan et al. 2011). MNPs are highly sensitive and selective for diagnostic purposes, and no problem of magnetic background exists with the use of these NPs as biological samples do not exhibit any virtual magnetic background. A rapid and sensitive nanosensor based on the conjugation of biotin-labeled aptamer with the streptavidin-coated iron oxide NPs has been constructed for cancer detection. Aptamer possesses the ability to specifically recognize and bind the target cancer cell, whereas large surface area of MNPs can easily accommodate large number of aptamers that could help the clinicians to accurately identify the cancerous cells at single-cell level or molecular level (Bamrungsap et al. 2012).

6.3 Therapy

6.3.1 Cancer Treatment

Mostly, 99% of the drugs and many methods like surgery or chemotherapy are used to fight with the cancerous cells but they are unable to reach to the cancer cells or decipher toxicity levels into the healthy cells (Sunderland et al. 2006). One of the basic causes for cancer to occur in the body is over-expression and overactivity of certain receptors, antigens, growth factors, or hormones such as epidermal growth factor receptor, vascular endothelial growth factor, integrins, transferrin, folate receptors, etc. The cancerous cells exhibit unique properties which can be exploited by different NPs. The treatment of cancer cells now involves the targeting of specific biomarkers or antigens present on their cell surface, so that the cells apoptize at the same place rather than getting migrated to different places and causing damage to the normal body cells. Researchers are trying to design NPs in such a way so as to conjugate various active targeting moieties to modify the surface of nanomaterials.

Metallic NPs possess properties like selective in nature, biocompatible, and smaller size; being amenable to be surface modified has been extensively explored for their usage in cancer treatment. The small size of NPs enhances their surface area and thus serves to be more effective. AgNPs synthesized using culture supernatants of *Bacillus funiculus* hampered the growth of MDA-MB-231 (human breast cancer cells) in a concentration-dependent manner. The cytotoxicity caused due to activation of the lactate dehydrogenase enzyme and caspase-3 and further formation of reactive oxygen species (ROS) eventually lead to stimulation of apoptosis and resulted in nuclear fragmentation (Gurunathan 2014). In another report, methotrexate (MTX), an inhibitor of dihydrofolate reductase, was conjugated to AuNPs to prepare MTX-AuNP resulting in higher accumulation of MTX in the tumor cells which in turn showed greater cytotoxicity in tumor cell lines. It was also found that the tumor cell growth was suppressed in mouse ascite model of Lewis lung carcinoma (LL2) on administering MTX-AuNP conjugate as compared to free MTX (Chen et al. 2007). CuNPs of size 4–5 nm are also known to exert cytotoxic effects in a dose-dependent manner toward human histiocytic lymphoma cells, U937 and human cervical carcinoma cells, Hela cells by inducing apoptosis through the generation of singlet oxygen scavengers (Jose et al. 2011). In another study, CuO NPs cause the greater cytotoxicity to A549 cell lines as compared to their bulk counterparts. The results suggested that CuO NPs may cause cellular death through autophagic pathway as the autophagic biomarker named LC3 II increased after A549 cells were exposed to CuO NPs (Sun et al. 2012). The apoptotic effect of CuNPs is caused by the development of ROS and further causing the disruption of mitochondrial membrane (Sankar et al. 2014). A general strategic representation of how the NPs play a role in targeting and treatment of diseased organ through drug delivery is shown in Fig. 3.

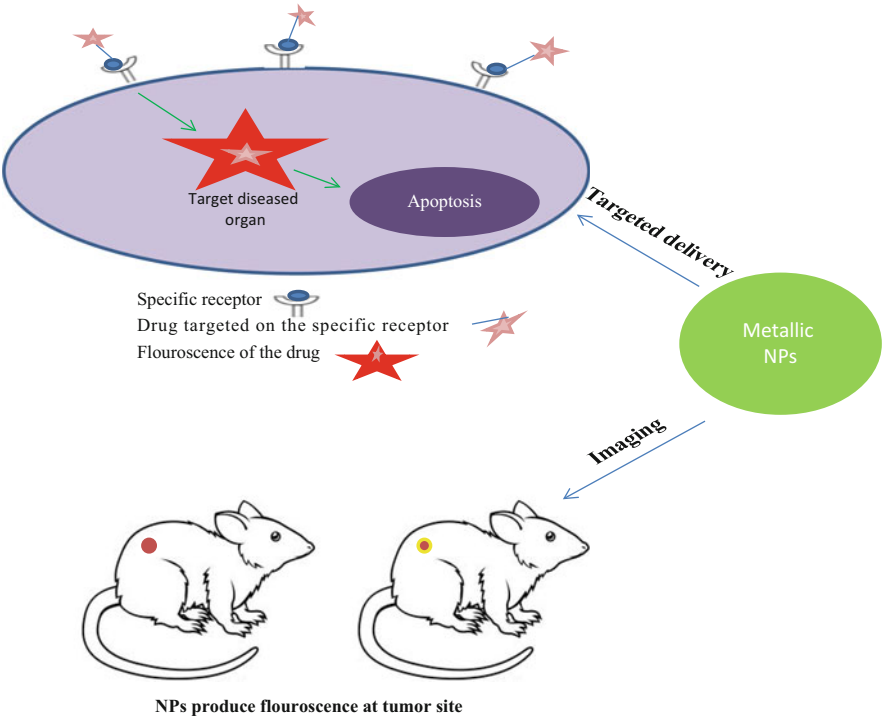

Fig. 3 Schematic representation of applications of metallic NPs in drug delivery and biomedical imaging for disease diagnostics

Johannsen et al. (2007) has reported to give an injection of magnetic iron oxide NPs at a particular dosage for 1 hour once in a week for a period of 2 months into the patients having recurrent prostate cancer and found that NPs (20 nm) could easily enter the cancer cells and produced heat under magnetic fields of 50–100 kHz causing the photothermal death of the cells. In another study, SPION micelles were synergized with β-lapachone (β-lap), an anticancer drug reported ten times greater ROS stress developed in cells exposed to β-lap-along with SPION-micelles as compared to cells treated with β-lap alone, resulting in increased cell death. These SPION micelles along with ROS generating drugs can serve as novel NPs for the cancer treatment (Huang et al. 2013).

6.3.2 Disease Treatment

Metallic NPs have been used in administration of drug due to their properties like small size, longer retention in the body, and ability to penetrate the cell membrane with drug encapsulated in the core. AgNPs play a deterministic job to treat diabetes. In an attempt to prove AgNPs as a beneficial candidate to lower down the blood

glucose level, streptozotocin-induced diabetic mice were administered orally with AgNPs at a dose of 10 mg/kg and found that there was reduction of 68.2% in the blood glucose level. The results demonstrated that AgNPs could increase the serum insulin level by 3% in the diabetic mice in comparison to the diabetic mice treated with insulin. The glucokinase activity was enhanced by 25.8%, and there was a significant increase in the expression of IRA and GLUT-2 in the diabetic rats elucidating the role of AgNPs as antidiabetic agent (Alkaladi et al. 2014). For the treatment of rheumatoid arthritis (RA), a complex of hyaluronate-AuNPs/tocilizumab (HA-AuNP/TCZ) was given which illustrated their therapeutic role. AuNPs bind to vascular endothelial growth factor (VEGF), whereas TCZ is immunosuppressive in nature which acts against interleukin-6 receptor and HA has lubricating properties (Lee et al. 2014).

6.3.3 Radiotherapy

The intensity of the ionizing radiations falling on the tissue undergoes a variety of molecular processes like Crompton effect and Auger effect. These effects create a change in the state of electrons which causes release of energy causing damage to the living cells. Water is the main component of the cells, and when the cells are exposed to these radiations, they generate aqueous free radicals which further react with the cellular components like proteins, DNA, RNA, etc. and hinder the normal metabolic processes of the cell. This occurs in both diseased and normal cells. So, approaches are being developed which may target and destroy only the diseased cell and render normal cells to be harmless by the development of nontoxic radioprotectors (Nair et al. 2001; Upadhyay et al. 2005).

AgNPs were intricated with alpha-lipoic acid (LA, an antioxidant molecule) and was stabilized using 1% pluronic F-127. They exhibited free radical scavenging action which was revealed by the DPPH assays in vitro. The anti-inflammatory activity was speculated in paw models of edema in mice. The results demonstrated that the use of AgNP-lipoic acid complex protected the mice from weight loss when exposed to gamma radiation and is effective on tumor growth delay when given to the mice before any exposure to the gamma radiation. Their antioxidant, radioprotecting, and anti-inflammatory properties revealed that they helped in protecting normal tissues and did not obstruct the delaying effect on the tumor growth (Ramachandran and Nair 2011). The effect of AgNPs with their interaction with gamma radiation (6 MeV gamma photons) in breast cancer has been studied. NPs serve as photo-absorbing agents which further increase the competence of radio waves in cancer therapy. Their mode of action relied on the fact that AgNPs have high mass energy absorption coefficient which in turn generated free radicals that caused damage to cancerous cells by denaturing the DNA. Their usage has helped in detecting and targeting the cancer cell simultaneously (Salih 2013). Gold has an outstanding effect in absorbing X-rays. AuNPs hold many properties like inertness, biocompatible, and low osmolality. AuNPs when administered to the tumor cells may lead to a higher dose to the cancer cells in comparison to the normal cells when

irradiated (Hainfeld et al. 2008). Iron-gold core-shell MNPs Fe@Au NPs were synthesized which could be used as a tool for imaging as well as targeting the tumor area by applying the magnetic field. Fe@Au NPs of mean size 70 nm were synthesized and checked in human breast cancer cell line (MCF-7). It was found that the malignant cells treated with NPs had increased the effectiveness of the radiation, thereby causing the survival of only 33% cells. These NPs did not show any cytotoxic effects (Manjili et al. 2014).

6.4 Theranostics

Theranostics is an emerging field in nanomedicine which takes advantage of NPs for the dual purpose of imaging and therapy of disease. The nano-platforms have the capacity to load the molecules which carry imaging and therapeutic functions. These multifunctional nanostructures used for disease diagnosis and drug delivery and to monitor the response of a given therapy will attract the dawning era of personalized medicine. Researchers throughout the world are engaged in the field of theranostics to construct these kinds of function-integrated new agents. To develop newer nano-based theranostics agents, it is needed to understand the surface chemistry of NPs so as to easily conjugate or load the other moieties having pharmaceutical role.

Metallic NPs play a great job in theranostics due to the convenience to manipulate their shape. Moreover, they possess the fluorescent properties used for imaging and properties to generate oxidative stress due to which these can act as anticancer agents for therapeutic purpose playing the dual role of theranostics. Advances in nanotechnology have led its way to produce multifunctional hollow gold nanospheres which can act to generate photo-acoustic signals and also help to induce photothermal ablation for therapeutics. Gold nanospheres conjugated to RGD peptide for targeting to integrin receptors that are highly expressed on glioma and angiogenic blood vessels have shown that photothermal ablation therapy increased the survival rate of tumor-bearing mice (Lu et al. 2011). In another study, the researchers have investigated that AuNPs with PEG surface modification and then combined with radiotherapy possess diagnostic and therapeutic potential for sarcoma. They suggested that PEG-AuNPs in combination with radiotherapy enhanced CT imaging, whereas radiotherapy helped to induce tumor tissue damage by increasing unrepaired DNA damage. AuNPs were potentially used for improving target imaging and radiosensitization of tumor while minimizing dose to normal tissues thus providing more chances of survival to animals under experimentation (Joh et al. 2013). CuNPs, other kind of metallic NPs, also come into picture for theranostics. Zhang et al. (2015) have prepared high-density CuNPs which provided fourfold higher signals as PAT contrast agents in NIR region than that of blood. $\alpha_v\beta_3$-targeted CuNPs effectively delivered Sn 2 lipase-labile fumagillin prodrug, a potent anti-angiogenic therapy, under in vivo conditions which has set an example of a systemically targeted drug delivery therapy with a PAT agent.

MNPs are a generation of advanced NPs which act both as imaging and therapy playing a role in theranostics. Wang et al. (2014) have developed a system based on iron oxide NPs for photodynamic therapy (PDT) and imaging of head and neck squamous cell carcinoma. PDT is an alternate treatment to various cancers other than chemotherapeutic agents which involves the activation of photosensitizer through the use of light of a specific wavelength which further interacts with molecular oxygen to produce singlet oxygen and ROS. These species lead to tumor death mediated by apoptosis and necrosis. These iron oxide NPs combined with a PDT drug, Pc 4, and NPs were first conjugated with a fibronectin-mimetic peptide (Fmp), specific to integrin β1. Their results showed that targeted Fmp-IO-Pc4NPs induced greater inhibition of tumor cells when compared to non-targeted IO-Pc 4 NPs in which iron oxide NPs were not conjugated to Fmp. This developed system of nano-therapeutics has a great potential to serve as MRI contrast enhancement agent due to the presence of iron oxide NPs, whereas the presence of PDT drug helps in therapy of cancer cells.

7 Conclusions

The nanocompound synthesis mediated by microbial species including bacteria, fungi, and actinomycetes is of great importance in the recent times as the biological synthesis route is simple, environmental-friendly, and inexpensive when compared to chemical and physical methods used for NP development. The requirements of costly reagents and post-processing needs/instruments to form stable NPs can easily be replaced by the use of alternate approach involving microorganisms, which in turn will help in decreasing the cost of synthesis operations, as the microorganisms are widely abundant in various natural habitats and can easily be cultured and maintained on large scale. The nanocompounds like metallic NPs (Ag, Au, Cu, iron oxide, etc.) are synthesized by the microbes by adopting mainly two different pathways of either intracellular or extracellular. The extracellular approach used for the synthesis of nanomaterials is advantageous over the intracellular approach as it does not involve any labor during downstream processing for the extraction of NPs from the interior of microbial cells and is used for the large-scale synthesis of NPs. The metallic nanocompounds synthesized possess many extraordinary properties (physical, chemical, optical, thermal, and mechanical) as compared to their bulk counterparts. Moreover, the nanocompounds synthesized by microbes are biocompatible and biodegradable in nature. Due to these astonishing properties, the biogenic nanocompounds play a role in medical applications. SPR and SERS effects possessed by metal NPs make their use in certain diagnostics techniques for the detection of deadly diseases like cancer. These nanocompounds can even be used for the treatment of certain diseases as therapeutic agents. These biosynthesized NPs possess antimicrobial activity due to which these particles can be used in certain medical instruments to prevent microbial infections and in tissue engineering applications. The use of these nanocompounds can bring a great positive change in wide spheres of life.

8 Future Perspectives

The large-scale synthesis of nanocompounds using bacteria is exciting because it does not need any hazardous, toxic, and expensive materials for synthesis and stabilization processes. It seems that by optimizing the reaction conditions and selecting the best bacteria, these natural nanofactories can be used in the synthesis of stable NPs. Drawbacks associated with the biosynthesis of nanocompounds using bacteria are tedious purification steps and poor understanding of the mechanisms. The main challenges faced in the synthesis of nanocompounds using bacteria are to control the shape and size of the NPs and to achieve the monodispersity in solution phase. Another challenge in synthesis of phytocompounds using bacteria is large-scale scaling up for industrial production. Furthermore, little is known about the mechanistic aspects, and information in this regard is necessary for economic and rational development of nanocompound synthesis using bacteria.

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Microbial Source of Melatonin and Its Clinical Aspects

Sanjay Kumar, Brendan Patrick Mulligan, Shreesh Ojha, and Alex Tinson

Abstract Melatonin chemically known as *N*-acetyl-5-methoxytryptamine is a small physiological compound of diversified functions. Melatonin has been found in phylogenetically different taxa of bacteria, unicellular eukaryote, microalgae, plants, fungi, and animals. Identification of melatonin in many of these microorganisms is missing, and its function is rarely known. Although, melatonin in microorganisms is not essentially involved in circadian process, rather, it exhibits antioxidant property also and may protect chlorophyll pigment to damage from stress and free radicals. Mostly, the pathway for melatonin synthesis in microorganisms shows similarity with vertebrates. Investigation on melatonin in some organisms allows more concrete discussion about their possible role. The various functions of melatonin in human including sleep and regulation of circadian rhythm has been well characterized. Here, we have focused on the mechanism of immune regulatory, antioxidant, and scavenging property of melatonin during pathogenesis caused by fungi, bacteria, and virus. This article will provide a view on microbial sources and possible therapeutic aspects of melatonin in future.

Keywords Melatonin • Circadian process • Immune regulation • Antioxidant property • Scavenger of free radicals

1 Introduction

Melatonin is key signal molecule, widespread all through plants and animal kingdoms and likely exists in every living organism (Dubbels et al. 1995; Manchester et al. 1995; Tan et al. 2007; Byeon et al. 2014; Park et al. 2013; Reiter et al. 2013).

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Therefore, today melatonin is considered as universal molecules exist in most living being on earth (Tan et al. 2012). Even though information on melatonin is widely absent in some important taxa of organisms, particularly in Archaea, bryophytes (especially mosses and ferns), gymnosperms, Porifera, Annelida, Arthropoda (mainly Chelicerata), and Echinodermata. Very few reports are available on melatonin in some other large groups of taxa. The existence of melatonin in different taxa manifests a high evolutionary emergence of this molecule. Thus, in such diverse group of phylogenetically isolated organisms, it is difficult to hypothesize that this molecule plays the equal physiological roles everywhere, partially not as signaling. However, opposite to the potential heterogeneity in signaling process, such variations do not occur in physicochemical characteristics of this compound. Therefore, its antioxidant and amphiphilic behavior is of great significance in many aerobic organisms from mostly distant taxa (Hardeland et al. 1995). Melatonin is a thoroughly studied endogenous substance. It involves in variety of constructive processes like regulation of cell cycle inside the cells of plant and animals (Reiter et al. 2006). Although it has been generously explored, the entire behavior of melatonin is acquiring constantly strong scientific enthusiasms for researchers. The focus of this study is to highlight the confined sources of this compound in different microorganisms and its clinical importance including growing them of eagerness.

2 Melatonin Production in Different Microorganisms

The melatonin has been observed in several species of non-vertebrates, but in few of them, it has been associated with circadian rhythm. In *Gonyaulax polyedra*, dinoflagellate melatonin level raises directly just after the start of dark condition, and the process of rhythmicity persistence remains consistent with darkness (Hardeland et al. 1995). *Euglena gracilis's* earliest derived eukaryotic cells also exhibits nocturnally peaking diurnal rhythms of melatonin, whereas in numerous unicellular blue-green algae like *Dunaliella tertiolecta* and in many *Chlamydomonas* species, the melatonin has been linked with nocturnal rhythmicity (Hardeland et al. 1995). Remarkably, melatonin has been detected in *Rhodospirillum rubrum*, an aerobic photosynthetic prokaryotic bacterium (Manchester et al. 1995). Further, the production of melatonin has been studied broadly in *Erythrobacter longus*, an aerobic photosynthetic bacterium (Tilden et al. 1997). This bacterium was first identified by Sato et al., and 20 more strains has been isolated (Shimada 1995). Unlike anaerobic photosynthetic bacteria, these bacteria produce photosynthetic pigment bacteriochlorophyll a (Bchl a) only in aerobic environment (Shimada 1995). It has been speculated that under alternating dark and light phase, one photosynthetic aerobic bacterial strain produces higher yield of biomass as compared to consistent dark and light phases (Yurkov and Beatty 1998). Thus this process of alternating night and day cycles is extremely similar to the circadian rhythms in most plants and animals. A photosystem II protein has been shown to participate in circadian rhythms in blue-green algae and mutant strains of cyanobacteria (Liu et al. 1995). Tilden et al. (1997) examined the melatonin

production as influenced by light in *Erythrobacter longus* bacteria, and its concentration has been found higher in darkness. The differences of melatonin concentration between day and night cycles of circadian rhythm satisfy in such a way whether it is produced at a higher rate in dark phase or their synthesis reduced in day condition may be because of photo catalytically destroyed in presence of light. Although, the task of melatonin is not fully investigated in prokaryotes so far. Previous studies have suggested that melatonin may protect photosynthetic pigment Bchl during its synthesis. The stability of Bchl a is restricted to dark phase when it is situated in the cell membrane of *E. longus*; however, it is also stable in light condition (Shioi et al. 1995). Thus, this molecule could protect Bchl additionally against free radicals by scavenging them, because several free radical species are produced during synthesis of Bchl a, which may damage it. This property of melatonin would suggest that melatonin might have produced because of this or similar in association with photosynthesis.

Even though the melatonin's role in rhythmicity process has been studied in several species of non-metazoan, this is not a universality for all. Yeast strain of *Saccharomyces cerevisiae* produces high yield of melatonin and related other compounds like methoxyindoles, but there is no sign of circadian rhythm existence (Sprenger et al. 1999). Melatonin is an indolamine, and its concentration has been found to increase in fermentation process during wine production (Rodriguez-Naranjo et al. 2011). Therefore, it seems to play a crucial role in yeasts. Many microorganisms absorb tryptophan from media as nitrogen source and produce several metabolic products as tryptophol or biogenic amines and indoleacetic acid. The tryptophan is an important precursor molecule for melatonin formation in biosynthetic pathway. The capabilities of yeast to produce indolamines have been evaluated with flavors of wine (Arevalo-Villena et al. 2010). The quantity of melatonin has been directly correlated with amount of yeasts consumed during production of grape juice (Arevalo-Villena et al. 2010). Nonetheless, very few data are reported on melatonin production in fermented products. The biogenic amines are extensively studied biomolecule because it affects the organoleptic property of food and substances, which influences health negatively (Silla-Santos 1996; Ancín-Azpilicueta et al. 2008). Yet purposes in yeast strains to produce melatonin have not been studied earlier, although it has been related positively to health (Agorastos and Huber 2011; Motilva et al. 2011). Recently, the comparative analysis on melatonin production has been performed under different parameters of growth and fermentation process. Here, author has shown that different strains of yeasts produce melatonin in variable concentrations (Rodriguez-Naranjo et al. 2012). The media components for the growth of yeasts are important for melatonin formation during fermentation process. The tryptophan in media has been directly associated with production of melatonin during growth of yeasts. The increased concentration of tryptophan enhances the final melatonin yield. Thus, melatonin could be considered as growth signals for the yeast growth.

3 Synthesis and Regulation of Melatonin

Basically, melatonin was seen as unique pineal gland secretions in vertebrates, called a neurohormone. In vertebrate, the melatonin is formed exclusively from tryptophan, an essential amino acid. Initially, little information was available on melatonin biosynthesis in organisms other than vertebrates; in yeast the pathway seems to be similar to the synthetic route and enzymes described in vertebrates (Sprenger et al. 1999). Recently, in non-metazoans, for example, bacteria, protistans, fungi, algae, and plants, melatonin is formed by shikimic acid pathway, where tryptophan is first synthesized using D-erythrose-4-phosphate, phosphoenolpyruvate, and carbon dioxide (Bochkov et al. 2012). Therefore, melatonin synthesis in these organisms is not limited by tryptophan as an initial source, and this leads to exceptionally higher yield of melatonin generally better than those in higher vertebrates (Fuhrberg et al. 1997; Chen et al. 2003; Hardeland et al. 2007). In vertebrates, melatonin synthesis has been broadly studied (Hardeland 2010). It starts with tryptophan as an initial molecule and leads to final product of melatonin using four different enzymes in four sequential steps. Enzyme tryptophan hydroxylase hydrolyzes tryptophan into 5-hydroxytryptophan (5HT) using water molecules in initial step. In the next step, 5HT is converted into serotonin with removal of carbon dioxide with the help of enzyme aromatic amino acid decarboxylase. Then serotonin is acetylated by serotonin acetyltransferase, and final product melatonin is formed from *N*-acetyl serotonin by hydroxyindole-*O*-methyltransferase (detailed in Fig. 1).

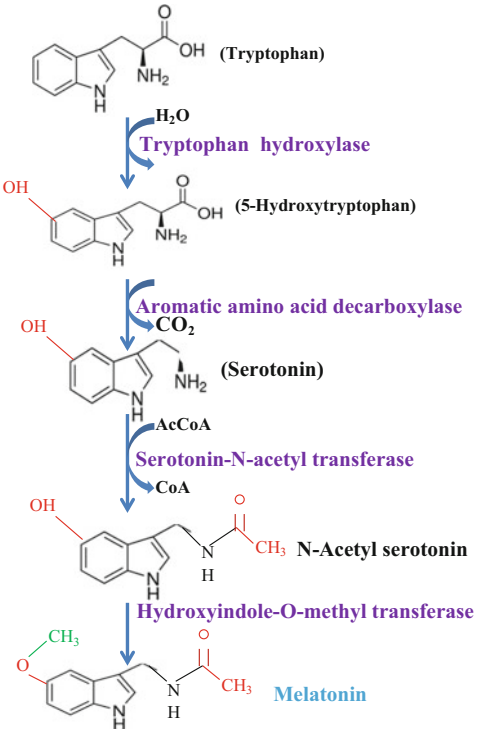

Fig. 1 Biosynthetic pathway of melatonin

The melatonin formation in pineal gland is controlled by suprachiasmatic nucleus (SCN), situated in the hypothalamus, and catches the signals from the retina via retinohypothalamic path. Melanopsin, present in retinal ganglia, is connected through nerve cells (Berson et al. 2002). These nerve fibers finally terminate to pinealocytic pigments and operate the melatonin synthesis by discharging norepinephrine hormones, which bind to receptors of pinealocytic pigments and stimulate the cAMP through α-G protein subunit (Moore 1997). The higher cAMP enhances the production of enzymes involved in melatonin synthesis. As melatonin is synthesized in the pineal gland, it is immediately secreted in blood or cerebrospinal fluid and metabolized in the liver.

4 Functions of Melatonin

4.1 Association of Melatonin with Cancer

In experimental rat, melatonin delays the initiation of tumorigenesis. Several carcinogenic compounds bind to DNA and form DNA-bound carcinogenic complex, which accumulates in the cells and causes mutation by altering the DNA permanently. Melatonin has been shown to bind these DNA-bound carcinogens and overcome the mutations and prevent the cancer initiation. Melatonin has capacity of scavenging free radicals and detoxifies carcinogens through activation of glutathione and related antioxidative pathways. It has been demonstrated that it stimulates the repair of damaged DNA, which was harmed due to formation of DNA carcinogenic complex, and protect normal cells (Blask et al. 2002). The cancer cell proliferations have been inhibited by melatonin in vitro condition using experimental model of neoplasia. This may be achieved by suppressing the cell progression at particular point in cell cycle. In few human cancer cells, melatonin performs as a differentiating factor and nullifies their metastatic and invasive property through alterations in adhesion molecules (Blask et al. 2002, 2005). Melatonin at physiological conditions regulate the linolenic acid (LA) uptake and its metabolism in cancer cells. LA is the most prevalent omega-6 polyunsaturated fatty acid, usually rich in diet in Western countries, which promotes cancer proliferation in human and murine (Sauer et al. 2001).

Melatonin interacts with many intracellular proteins like calmodulin, nuclear receptor proteins, and receptor located in membrane (Dubocovich and Markowska 2005). Three receptors of melatonin have been identified by the International Union of Basic and Clinical Pharmacology. Melatonin receptor 1 shows high affinity to melatonin, where melatonin receptor 2 and melatonin receptor 3 are low-affinity receptors (Dubocovich and Markowska 2005). Melatonin receptors 1 and 2 belong to GPCR protein class because of their amino acid similarities (Reppert 1997). Both melatonin receptors 1 and 2 inhibit adenyl cyclase and cyclic AMP (cAMP) followed by reduced uptake of linolenic acid. Lipoxygenase enzyme oxidizes

linolenic acid to 13-hydroxyoctadecadienoic acid (13-HODE), a strong source of energy for tumorigenesis. This reduced uptake of linolenic acid by melatonin is considered as its antiproliferative activity against cancer cells. Melatonin receptor 3 is the member of X-linked orphan G-protein-coupled receptor (Ivanova et al. 2008) and identified as a quinone reductase 2 (QR2), which helps in detoxification of carcinogens. The induced gene expression of QR2 is correlated with reduced susceptibility to cancer growth (Mailliet et al. 2005; Tan et al. 2007). The antiangiogenic properties of melatonin have been accomplished by inhibition of many growth signals like epidermal growth factor, vascular endothelial growth factor and insulin growth factor 1. All growth signals are strong mitogens and stimulator of cancer angiogenesis (Park et al. 2010). The research on antiangiogenic property of melatonin is of great significance on its future clinical application.

4.2 Antibacterial Property of Melatonin

The several studies have been carried out on melatonin, but its antimicrobial property in vitro is investigated in very few researches. Melatonin is a natural substance with no known toxicity and protection against harmful free radical species and nitric oxide in animal (Reiter 1997; Reiter et al. 1997a, b). However, no reports are available that melatonin has direct effects on viability of pathogenic bacteria. Melatonin has been shown to enhance the activity of isoniazid (INH) against tuberculosis. INH is frontline antituberculosis drug and may depend on the generation of free radical species for their side effects. The rapid development of INH drug resistance with their adverse side effects in patients needed some agent to potentiate INH concentration and protect the patient for their adverse side effect. This is accomplished by the ability of melatonin to form stable radicals and may initiate the activation of INH by adding more melatonin. In addition, melatonin is a hydrophobic molecule and has been demonstrated to locate itself in the mycobacterial cell wall and destabilize the cell wall components and enhance the permeability of membrane for INH molecule (Barrett et al. 1994).

Many metal ions like zinc, iron, and copper are present in cytoplasm of organisms in free form. Melatonin has strong metal-binding capacity (Limson et al. 1998). It binds to these free metal ions and reduces the cytoplasmic availability for pathogenic microorganisms, strongly dependent on free metal ions for their growth in host (Ward et al. 1996). Several mechanisms have been reported for the possible antibacterial property of melatonin. The iron is absolutely essential for bacteria. Melatonin consumes free iron metal inside the cytoplasm, makes bacteria deprived of iron ions, and inhibits their growth. Gulcin et al. (2003) have reported the effective chelating property of melatonin. This feature of melatonin has been correlated with its antioxidant property by depleting free transition metals essential for bacterial growth.

In recent report, melatonin has been shown as antibiotics to inhibit gram-negative as well as gram-positive bacteria (Tekbas et al. 2008). The author used

very problematic bacteria in this study as these pathogens develop resistant strains against antibiotics in nosocomial infections (Chastre and Trouillet 2000; Huletsky et al. 2004; Jeong et al. 2006). In this study, the author observed as melatonin was more powerful in killing gram-negative bacteria than gram-positive one, because the cell membrane is rich in lipopolysaccharide and protein glycopeptides and also massive quantity of lipid in gram-negative strains. Bacterial membrane is full of phospholipids, and melatonin reduces the uptake of linolenic acid and regulates the total fatty acid balance in breast cancer cell lines in human (Tan et al. 2007). This feature of melatonin may be associated with regulation of fatty acid balance in bacteria cell membrane. Melatonin molecules have long shelf life, very safe, less side effects, and less expensive as compared to other conventional drugs and thus can be used to overcome the several bacterial infections.

4.3 Antifungal Property of Melatonin

The expected function of 5-hydroxytryptamine (serotonin) against fungal infection has been suggested by several studies. 5-hydroxytryptamine is available in blood platelets, which has the ability to bind hyphae of *Aspergillus fumigates* and kill them (Christin et al. 1998). Further, Lass-Flörl et al. (2003) have found that serotonin destroys the conidia and hyphae of *Aspergillus* spp. in vitro also. Several other antimycotic drugs (miconazole and econazole) have been observed to interfere with platelets by inhibiting the uptake of serotonin and could contribute to the synergistically defense action against fungal infections (Helmeste et al. 1998). The study by Lass-Flörl et al. (2003), serotonin has been found to act as antimycotic agent against *Candida* species in a two-step reversible attenuation, if incubation is for a long time and irreversible changes finally lead to loss of viability. Serotonin is one of the important intermediates in the melatonin biosynthesis pathway (Fig. 1). Thus melatonin could be correlated with its antimycotic action against fungal pathogenesis.

Melatonin receptors in nuclear and cell membrane have been broadly studied, and the interaction between melatonin and its receptors has been correlated with its immune function regulation (Garcia-Maurino et al. 2000; Reiter et al. 2000). Although the immunomodulation by melatonin has been usually reported in many studies, anti-inflammatory action of melatonin has yet to be fully understood. Many reports have stated that melatonin has an anti-inflammatory property (Sutherland et al. 2002; Jiménez-Caliani et al. 2005; Wang et al. 2005). Yavuz et al. performed the antifungal property of melatonin in rat sepsis infected with *Candida albicans*. In this study, reduced expression of IL-6 was observed in sepsis-diseased rat group treated with melatonin only as compared to same rat group treated with amphotericin (an antifungal drug). The author also studied that expression levels of adhesion molecules and TNF-α were found to reduce in melatonin-treated septic rat group as compared to rats treated with only amphotericin B. This reduced expression profile of interleukin-6, tumor necrosis factor-α, and adhesion molecules

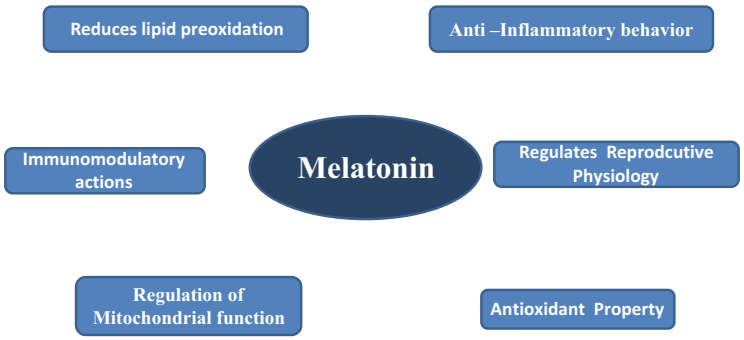

Fig. 2 Pleiotropic actions of melatonin

lessened the improvement time of sepsis disease in animals infected with *Candida* species. The expression profile of markers such as interleukin-6, tumor necrosis factor-α, and adhesion molecules is highly increased with severity of infection and in whole period of the disease (Filler et al. 1996; Giese et al. 2000; Cannom et al. 2002; Reinhart et al. 2002). Thus, melatonin could be considered as antimycotic molecule, which could be supplemented with other antifungal drugs against the systemic *Candida* infection because of its immune regulatory property (Fig. 2).

4.4 Melatonin in Reproduction

Several works have been carried on melatonin in terms of regulation of testicular function. The strong binding site for melatonin has been identified in testes of rat, which shows the existence of melatonin receptors in testis (Vera et al. 1996). It is known that maturation and storage of spermatozoa occurred in the epididymis. The binding sites for indomelatonin have been recognized in the epididymis of rat (Shiu et al. 1996). The male testosterone interacts with indomelatonin in the epididymis of rat and reversed the binding site of specific 2-[^{125}I] indomelatonin in the epididymis in rat, which overcomes the inhibitory action of melatonin on Leydig cells (Shiu et al. 1997). Gene expression level of melatonin receptors 1 and 2 in epididymal epithelial cells of rat suggested that melatonin could be associated with physiological process like maturation of spermatozoa in epididymis (Shiu et al. 1997). High-affinity melatonin receptor 1 protein has been detected in cytosol of epithelial cells of prostate gland in human (Laudon et al. 1998), which conferred that receptors are important in regulation of melatonin during development of prostate (Gilad et al. 1996). In another recent study, melatonin has been proposed to control the expression and function of gonadotropin-induced hormones in gonads, which helps in testosterone release in starling animals in Europe (McGuire et al. 2011).

The serum melatonin level is higher during pregnancies than normal women (Nakamura et al. 2001). The melatonin concentration has been found to rise in 28 weeks of gestation in twin pregnancy as compared to singlet one (Nakamura et al. 2001). Melatonin is a small molecule, moved from mother to fetus with blood through the placenta. In animal, melatonin concentration is found to be absent in case of pinealectomy and concludes that it is exclusively secreted from the pineal gland. Many receptors of melatonin have been detected in human fetal suprachiasmatic nucleus (Thomas et al. 2002). The suppression of melatonin has been observed to affect the expression of melatonin receptor 1 and clock gene (Torres-Farfan et al. 2006), which confers it is key molecule in regulation of circadian rhythm.

Melatonin has been speculated to regulate parturition period in some mammalians. Usually, parturition takes place in daytime in rat. After removal of the pineal gland in rat, parturition does not occur in daytime, but as melatonin therapy applies, parturition patterns are observed in daytime (Takayama et al. 2003). The gene expression for melatonin receptors has been characterized in myometrial cells of both pregnant and nonpregnant female (Schlabritz-Loutsevitch et al. 2003), while progression of labor pain from myometrial dormancy, a valuable gap junction, has been detected (Salomonis et al. 2005). This gap regulates the contraction of myometrial cells to induce strong forces for labor pain. The physiological melatonin coordinates the affinity between gap junctions (Olecese 2007), which concludes that melatonin might be involved in progression of parturition.

4.5 Antiviral Activity of Melatonin

It has been speculated that melatonin regulates the immune function in physiopathological as well as physiological conditions (Maestroni 2001). This indolamine binds to T-helper cells (Th cells) and stimulates the immune tolerance (Maestroni 1995). Usually, viral pathogens cause immune suppression. Melatonin has been reported to protect the mice to die during encephalomyocarditis virus (EMCV) infection (Boga et al. 2012). EMCV causes paralysis and acute stress in mice and leads to death. Furthermore, it reduced the viremia and delayed pathogenesis in mice caused by Semliki Forest virus (SFV) (Ben-Nathan et al. 1995). SFV is an arbovirus that causes fetal encephalitis in mice and low pathogenicity in humans. LP-BM5 leukemia retrovirus infects female C57BL/6 mice and causes group of disorder, called murine acquired immune deficiency syndrome. These viruses suppress secretions of cytokines from T-helper cell 1 and increase vitamin E deficiency and peroxidation of lipid in mice. Melatonin administration in mice stimulated B and T cell proliferations and enhanced the T-helper cell 1 to secrete more cytokine. Melatonin has been also shown to counteract immunodepression in mosquito-borne encephalitis disease in mice caused by West Nile virus (Maestroni 2001).

Melatonin has been observed to stimulate the release of many cytokines from lymphocytes like IFN-γ, TNF-α, and IL-1 α, while it reduces the IL-2 and IL-4 production in mice (Valero et al. 2002). Melatonin forms cascade of immunoregulatory responses for antivirus, antiproliferative, and immunomodulatory for IFN-γ during viral pathogenesis (Bonilla et al. 1997). Other study has demonstrated that IFN-γ stimulates the production of melatonin and serotonin level in lymphocytes and macrophages (Miller et al. 2006). This stimulation could be associated with antiviral activity of melatonin, which may regulate viral load at onset of Venezuelan equine encephalomyelitis (VEE) infection. VEE infects human and horse. Mice infected with VEE causes hyper-excitation, coma, and paralysis, which results into death (Bonilla et al. 1997). The regulation of the inflammatory response, scavenging nature of the free radicals like reactive oxygen species, and related antioxidative property of melatonin are gaining a strong pharmacological significance to explore the mechanism for host pathogen reactions during viral pathogenesis and future consequences (Srinivasan et al. 2010, 2012). Although very few weakness of melatonin is short half-life, and their concentration varies with duration of light (Currier et al. 2000). Melatonin in humans at pharmacological concentrations has been seen as nontoxic even in case of neonatal period (Gitto et al. 2009). The entire outcome studied in in vitro and in vivo animals suggested the probable immunoregulatory and antioxidative role of melatonin during viral pathogenesis.

5 Conclusion

All facts discussed here manifest that melatonin is widely distributed in almost whole taxa of living organisms. Although it is produced by many microorganisms like aerobic photosynthetic bacteria, dinoflagellate, and algae, it shows the strong antioxidant property protects them during stress condition as well as its involvement in circadian rhythm. The insight and hypothesis are needed to understand its presence and diverse functions in genetically distant group of species. It reduces the lipid peroxidation and inhibits the cancer cell progression. The antioxidant, free radical scavenging, and immunomodulatory function of melatonin against the pathogens like bacteria, virus, and fungi deserve particular attention for its therapeutic potential in concerned disease in future.

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Major Source of Marine Actinobacteria and Its Biomedical Application

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Abstract Marine is a vital source of novel microorganisms, which does not exist in terrestrial environment. Actinobacteria is a large group bacterium which resides predominantly in marine sediments, seawater, and marine flora and fauna. It has been referred as valued sources of bioactive compounds. Literature survey revealed that these marine microbes produce a variety of secondary metabolites. Marine fauna and flora-associated actinobacteria are also anticipated to produce a variety of novel bioactive agents. Though studies on this area are in an infant stage, numerous studies have been concerning specific aspects of population and secondary metabolites of actinobacteria dwelling in terrestrial and marine sediment. So, further consistent examination of symbiotic marine actinobacteria for novel species and bioactive compound is essential, because it will not only offer us with much useful ecological evidence but will also open a path to procure useful bioproducts with a maximum hit rate. Among the bioactive compounds obtained from marine actinobacteria, ~39% were tested against cancer/cytotoxic activity followed by pharmacological value. In this review chapter, the secondary metabolites of marine actinobacteria especially associated with marine plants and animals and its biomedical applications are described.

Keywords Actinobacteria • Bioactive compounds • Pharmaceutical • Flora • Fauna • Streptomyces • Marine environment

1 Introduction

The existence and continuous increase of antibiotic-resistant pathogens in the environment caused life intimidating contagions and risk, which destabilized the present healthcare structures. With rapidly growing pharmaceutical market and increased drug-resistant infectious diseases, the demand for new secondary

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bioactive metabolites has also been stimulated. The terrestrial resources have been greatly explored, and thus researchers make great effort to obtain lead molecules from the marine ecosystem. Currently marine bacteria, fungi, and algae have gained significant status in the pharmaceutical trade as they exhibited antimicrobial, antiviral, antitumor, anticoagulant, and cardioactive properties. Though, the biologists have long been captivated to identify exclusive microbes and novel compounds from marine environment.

1.1 Actinobacteria

Actinomycetes are the beam-shaped Gram-positive bacteria with G+C content >55% (Goodfellow and Williams 1983). The actinobacteria were previously considered as an intermediate among bacteria and fungi but now verified as prokaryote (Das et al. 2008). Marine actinobacteria are the most significant group of prokaryotes industrially. Among these, streptomycetes are most remarkable group pharmaceutically, known for screening more than 10,000 antibiotics. Several novel actinobacteria have been reported from marine sources *Streptomyces luteireticuli* sp. nov., nom. rev., *S. luridiscabiei* sp. nov., *S. puniciscabiei* sp. nov. and *S. niveiscabiei* sp. nov., *Kitasatospora putterlickiae* sp. nov., *K. kifunensis* comb. nov., *Saccharopolyspora* sp. nov., *Actinopolyspora indiensis* sp. nov., and *S. kathirae* and *S. thulasea* sp. nov. (Sivakumar 2001; Groth et al. 2003; Hatano et al. 2003; Park et al. 2003; Dhanasekaran et al. 2005; Liu et al. 2005). *Pseudovibrio ascidiaceicola* sp. nov. was isolated from ascidians (sea squirts) (Fukunaga et al. 2006). *Euzebya tangerina* gen. nov., sp. nov., within the new family, order, and subclass Euzebyaceae fam. nov., was reported from the abdominal epidermis of *Holothuria edulis* which is commonly known as sea cucumber (Kurahashi et al. 2010).

2 Actinobacteria Associated with Marine Flora and Fauna

In the marine ecosystem three realms of life, eubacteria, archaea, and eukarya, exist. A few thousand bacteria and archaea have been recorded from marine; however, about 230,000 species of flora and fauna have been documented (Carlo and Heip 2014). Marine organisms are continually under tremendous selection pressure counting space race, predation, external fouling, and reproduction. The marine resources are nowadays widely studied because of numerous reasons. One of the reasons is 36 known living phyla exist on the world surface, 34 phyla of them were reported from marine environments with more than 300,000+ recognized species of animals and plants (Faircloth et al. 2004; Kijoa and Swangwong 2004). In this chapter actinobacteria associated with few marine flora and fauna and its bioactive compounds are described.

2.1 Actinobacteria Associated with Marine Flora

2.1.1 Mangroves, Seagrasses, and Seaweeds

Mangroves are prime intertidal bionetworks of the tropical and subtropical zones of the world. The actinobacterial genera such as *Agromyces*, *Cellulomonas*, *Gordonia*, *Microbacterium*, *Mycobacterium*, *Micrococcus*, *Rhodococcus*, and *Streptomyces* were isolated from mangrove roots in the Iriomote Island and were tested against different pathogenic bacteria and fungi (Takeuchi and Hatano 1999). Baskaran et al. (2012) have reported that a great diversity of actinobacterial populations exist in the mangrove phyllosphere. Several novel species named *Agromyces brachium*, *Agromyces rhizospherae*, *Agromyces luteolus*, *Gordonia rhizosphera*, *Lysinimicrobium mangrovi*, and *Micromonospora avicenniae* were reported from mangroves with various bioactivities (Takeuchi and Hatano 2001; Hamada et al. 2012; Li et al. 2013). Seagrasses are the marine angiosperms, occupying the tidal and subtidal marine environment. Cultivable and non-cultivable molecular approaches showed that seagrasses harbor rich bacterial diversity (Weidner et al. 1996). *Thalassia hemprichii* is associated with ten genera of actinobacteria. Totally, 43 16S rRNA gene base pairs were selected from the gene data bank to understand the diversity of mangrove endophytic actinobacteria, and the analysis of RDP assignment tools showed that all the actinobacteria belong to the order *Actinomycetales* including 11 families and 14 genera. Based on the 16S rRNA data in the GenBank, a diversity of seagrass-associated actinobacteria is relatively higher, including 15 families of three subclasses, viz., *Acidimicrobidae*, *Actinobacteridae*, and *Nitriliruptoridae* (Karuppiah et al. 2014). The symbiont from *T. hemprichii* displays the NRPS and PKS genes, which suggests that the actinobacteria might function as an antibacterial agent (Wu et al. 2012). Kumar and Krishnan (2012) have reported that *Streptomyces* is the leading genus in seagrasses, which shows effective antibacterial activity against multiple drug-resistant pathogens. Seaweeds harbor a diverse group of bacteria, depending on the season, species, and thallus structure (Bengtsson et al. 2010). Beneficial interactions between seaweeds and bacteria are exchange of nutrients and secondary molecules (Egan et al. 2013). Karuppiah et al. (2014) have analyzed the 16S rRNA sequences recovered from the GenBank, among them 22 genera within two orders of *Acidimicrobiales* and *Actinomycetales* were found to be associated with seaweeds.

2.2 Marine Fauna

2.2.1 Ascidians and Mollusks

Ascidians, a class of tunicates (phylum: Chordata), marine sessile filter feeders, are present in solitary and colonial forms (Shenkar and Swalla 2011). Actinobacteria, namely, *Arthrobacter*, *Brevibacterium*, *Curtobacterium*, *Gordonia*, *Kocuria*,

Micrococcus, and *Nocardia*, were reported from *Didemnum ligulum*, and *Didemnum* sp. might play an important role in ascidians as well as the isolates were recorded for its activity against various human pathogens (Menezes et al. 2010). Mollusks are a group of invertebrates that include more than 100,000 species (Haszprunar and Wanninger 2012). Biodiversity of mollusk-associated actinobacteria was preliminarily reported by Kurahashi and Yokota (2002). El-Shatoury et al. (2009) isolated strains that belong to ten genera of actinobacteria along with two unidentified isolates from *Donax trunculus* (Romanenko et al. 2008). Actinobacteria were dominant in the cone snails such as *Conus pulicarius*, *C. rolani*, and *C. tribblei* and included 16 different genera of 11 families with four common genera (*Brevibacterium*, *Gordonia*, *Microbacterium*, and *Streptomyces*) (Peraud et al. 2009). Based on the 16S rRNA data obtained from the GenBank, it is estimated that 34 genera within 16 families of the order *Actinomycetales* are associated with the ascidians. Among them, *Streptomyces* (93 sequences), *Micromonospora* (37 sequences), and *Nocardia* (13 sequences) were abundant in the ascidians. Phylogenetic analysis of the mollusk-associated actinobacterial sequences from the GenBank indicated that 28 actinobacterial genera within the order *Actinomycetales* were associated with the mollusks (Karuppiah et al. 2014).

2.2.2 Corals, Sponges, and Fish

Coral reefs support most biodiverse and biologically productive significant communities in the tropical and subtropical marine environments. Initially, culture-dependent investigation of coral-associated marine bacteria revealed the occurrence of actinobacteria in corals (Lampert et al. 2006). Sponges harbor microbes up to 40% of their total biomass by providing with favorable environmental conditions (Friedrich et al. 2001; Webster and Taylor 2012). The novel actinobacteria, *Janibacter alkaliphilus* and *Janibacter corallicola*, were isolated from the corals, *Acropora gemmifera* and *Anthogorgia* sp., respectively, which has been recorded for active compounds (Kageyama et al. 2007; Li et al. 2012). Actinobacteria obtained from these healthy corals have strong antibacterial activity, suggesting that these bacteria can protect the hosts from the pathogens (Nithyanand et al. 2010; Zhang et al. 2012). Karuppiah et al. (2014) reported that coral-associated actinobacterial community contains about 523 sequences in GenBank, which include 62 genera of 32 families within five orders, viz., *Acidimicrobiales*, *Actinomycetales*, *Nitriliruptorales*, *Solirubrobacterales*, and *Thermoleophilales*. Among the Porifera-associated bacteria, members of *Actinomycetales* are frequently sponge specific and a dominant producer of bioactive natural products. There are many bioactives isolated and screened for various pharmacological activities (Table 1). Many of them proved to be good drug molecules with significant results for preclinical and clinical studies. Studies using culture-dependent and culture-independent molecular methods confirmed that new, abundant actinobacterial accumulations are associated with marine sponges (Montalvo et al. 2005; Zhang et al. 2006; Abdelmohsen et al. 2014). According to literature survey,

Table 1 Bioactive compounds isolated from marine actinobacteria

Sources	Species	Compounds	Activity	Reference
Marine sediment/soil				
Sediment	*Streptomyces* sp. DOSMB-A107	2-Butanol, 2-nitroso-, acetate (ester), 1-hentetracontanol, 1-eicosanol, phenylethyl alcohol, 1-hexadecanol	Antimicrobial	Baskaran et al. (2016)
		Phenol, 2,4-bis (1,1-dimethylethyl)	Antioxidant; antimicrobial; anti-inflammatory; analgesic	
		Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-ergotaman-3′,6′,18-trione, 12′-hydroxy-2-′-methyl-5′-(phenylmethyl)-, (5′à)	Antimicrobial; anti-inflammatory	
	Streptomyces parvulus DOSMB-	Phenol, 2,4-bis-(1,1-dimethylethyl)	Antioxidant; antimicrobial; anti-inflammatory; analgesic	Baskaran et al. (2016)
		Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	Antimicrobial; anti-inflammatory	
		7,8-Epoxylanostan-11-ol, 3-acetoxy	Antimicrobial; anti-inflammatory; antiarthritic; antiasthma; anticancer	
		Dihydroergotamine mesylate	Antimicrobial; anti-inflammatory	
		1,2-Benzenedicarboxylic acid, diisooctyl ester, phthalic acid, diisodecyl ester	Antimicrobial; antifouling	
		Octadecane, 3-ethyl-5-(2-ethylbutyl)	No activity reported	
		Ethyl iso-allocholate	Antimicrobial; anti-inflammatory; antiarthritic; antiasthma; anticancer	

(continued)

Table 1 (continued)

Sources	Species	Compounds	Activity	Reference
	Nocardia dassonvillei	*N*-(2 hydroxyphenyl)-2-phenazinamine (NHP)	Anticancer; antifungal	Gao et al. (2012)
	Streptomyces sp.	2-Allyloxyphenol	Antimicrobial	Arumugam et al. (2010)
	Streptomyces sp.	Tirandamycins	Antibacterial	Carlson et al. (2009)
	Streptomyces sp.	Carbomycin	Antibacterial; anticancer	Hohmann et al. (2009)
	Streptomyces sp. Merv8102	Essramycin	Antibacterial	El-Gendy et al. (2008)
	Salinispora tropica	Salinosporamide A	Anticancer; antimalarial	Jensen et al. (2007)
	Streptomyces sp. CNQ-418	Marinopyrroles	Antibacterial	Hughes et al. (2008)
	Streptomyces arenicola	Arenicolides A–C mild	Cytotoxicity	Jensen et al. (2007)
	Streptomyces sp.	Daryamides	Antifungal; anticancer	Sivakumar et al. (2007); Asolkar et al. (2006)
	Streptomyces arenicola	Arenimycin	Antibacterial; anticancer	Asolkar et al. (2006)
	Streptomyces corchorusii	Tetracenomycin D	Cytotoxic	Adinarayana et al. (2006)
	Marinispora	Marinomycins A–D	Antimicrobial; anticancer	Kwon et al. (2006)
	Streptomyces sp.	Staurosporinone	Antitumor; phycotoxicity	Wu et al. (2006)
	Streptomyces nodosus	Lajollamycin	Antibacterial	Manam et al. (2005)
	Salinispora tropica	Salinosporamides B and C	Cytotoxicity	Williams et al. (2005)
	Streptomyces sp.	Glaciapyrroles	Antibacterial	Macherla et al. (2005)
	Streptomyces chartreusis	Chartreusin	Antitumor	Xu et al. (2005)

	Streptomyces psammoticus	Polyketide antibiotic SBR-22	Antibacterial	Sujatha et al. (2005)
	Novel actinomycete	Chloro-dihydroquinones	Antibacterial; anticancer	Soria-Mercado et al. (2005)
	Streptomyces chinaensis	1-Hydroxy-1-norresistomycin	Antibacterial; anticancer	Gorajana et al. (2005); Kock et al. (2005)
	Thermoactinomyces sp.	Mechercharmycins	Anticancer	Kanoh et al. (2005)
	Streptomyces sp.	Resistoflavin methylether	Antibacterial; anti-oxidative	Kock et al. (2005)
	Streptomyces sp. M045,	Chinikomycins	Antitumor	Li et al. (2005)
	Verrucosispora strain AB- 18-032	Abyssomicin C	Antibacterial	Riedlinger et al. (2004)
	Streptomyces aureoverticillatus	Aureoverticillactam	Antitumor	Mitchell et al. (2004)
	Streptomyces sp. BD21-2	Bonactin	Antimicrobial	Schumacher et al. (2003)
	Streptomyces sp.	Himalomycins	Antibacterial	Maskey et al. (2003)
	Streptomyces sp. KS3	Komodoquinone A	Neuritogenic	Itoh et al. (2003a)
	Streptomyces strain CNH-099	Neomarinone	Cytotoxic	Hardt et al. (2000)
	Streptomyces griseus, *Streptomyces halstedii*	Bafilomycin	ATPase-inhibitor of microorganisms and eukaryotic cells	Werner et al. (1984), Frädberg et al. (2000)
	Streptomyces sp.	Cyclomarin A	Anti-inflammatory	Renner et al. (1999)
	Streptomyces avermitilis	Avermectin	Antiparasitic	Burg et al. (1979)

(continued)

Table 1 (continued)

Sources	Species	Compounds	Activity	Reference
Soil	*Streptomyces coelicolor*	Chromomycins B, A2, A3	Antitumor	Lu et al. (2012)
	Streptomyces sp. BK 190	Elaiomycins B and C	Antitumor	Helaly et al. (2011)
	Streptomyces sp. GB-2	Sisomicin	Antibacterial	Lu et al. (2009)
	Streptomyces sp.	Bisanthraquinone	Antibacterial	Socha et al. (2006)
	Streptomyces lincolnensis	Lincomycin	Antibacterial, inhibitor of protein biosynthesis	Peschke et al. (2006)
	Streptomyces griseus	Frigocyclinone	Antibacterial	Bruntner et al. (2005)
	Streptomyces ochraceus, *Streptomyces bottropensis*	Trioxacarcins	Anticancer; antimalarial	Maskey et al. (2004)
	Streptomyces hygroscopicus	Hygromycin	Antimicrobial, immunosuppressive	Uyeda et al. (2001)
	Streptomyces rimosus	Tetracyclines	Antimicrobial	Hansen et al. (2001)
	Streptomyces galilaeus	Anthracyclines	Antitumor	Fujii and Ebizuka 1998
	Streptomyces griseus	Streptomycin	Antimicrobial	Egan et al. (1998)
	Streptomyces venezuelae	Chloramphenicol	Antibacterial, inhibitor of protein biosynthesis	Bewick et al. (1976)
Seawater	*Actinomadura* sp.	ZHD-0501	Anticancer	Han et al. (2003)

Sponge				
Hemiasterella spp.	*Streptomyces* sp. DOSMB-SP6	–	Antibacterial	Baskaran et al. (2016)
Tentorium spp.	*Streptomyces* sp. DOSMB-SP5	–	Antibacterial	
Tethyopsis sp.	*Streptomyces* sp. DOSMB-SP3	–	Antibacterial	
Acanthostrongylophora	*Micromonospora* sp. strain M42	Manzamine A, 8-hydroxy manzamine	Antiparasitic	Kubota et al.(2013)
Tethya sp. Mediterranean sponges	*Streptomyces* sp. *Streptomyces* sp. T03	Butenolide	Antitrypanosomal activity; antimicrobial; antifouling; estrogenic; serotonergic; anticancer; anti-HIV; antiparasitic activities	Cho et al. (2001), Shi et al. (2012), Husain et al. (2010), Michel et al. (2010), Pimentel-Elardo et al. (2003)
Aplysina aerophoba	*Micromonospora* sp. RV115	Diazepinomicin	Antitumor activity; antioxidant; antiparasitic	Campas (2009), Abdelmohsen et al.(2012)
Axinella polypoides	*Streptomyces axinellae* Pol001T	Tetromycins 1–4, tetromycin 1 (tetronic acid)	Antibacterial, antiparasitic activity, inhibition of protease	Pimentel-Elardo et al. (2009, 2011)
Hymeniacidon sp.	*Streptomyces carnosus* strain	Lobophorins C and D	Cytotoxic activity/cancer	Wei et al. (2011)
Craniella australiensis	*Streptomyces* sp. DA22	*N*-Phenylacetyl-L-tryptophan	Cytotoxicity	Huang et al.(2012)
Chondrilla caribensis f. caribensis	*Verrucosispora* sp. WMMA107	22′-deoxythiocoraline, thiochondrillines A–C, and 12-′-sulfoxythiocoraline (depsipeptides)	Cytotoxic activity	Wyche et al. (2011)
Demospongiae sponge	*Streptomyces* sp. SpD081030SC-03	JBIR-56 and JBIR-57 (3,5,6-trisubstituted 2(1H)-pyrazinones)	Cytotoxicity	Motohashi et al. (2011)
Suberites japonicus	*Actinomadura* sp. SBMs009	Bendigoles D–F	Cytotoxic activity	Simmons et al. (2011)
Aplysina aerophoba	*Streptomyces* sp.	Depsipeptide, valinomycin	Antiparasitic	Pimentel-Elardo et al. (2010)
Tedania sp.	*Streptomyces* sp.	Staurosporine	Antiparasitic	Pimentel-Elardo et.al. (2010)

(continued)

Table 1 (continued)

Sources	Species	Compounds	Activity	Reference
Haliclona sp.	*Streptomyces* sp. NBRC 105896	Teleocidin analog JBIR-31	Cytotoxic	Izumikawa et al. (2010a)
Haliclona sp.	*Streptomyces* sp. strain	Tetrapeptides JBIR-34 and JBIR-35	DPPH radical scavenging	Motohashi et al. (2010b)
Cinachyra sp.	*Streptomyces* sp. strain	Isoprenoids JBIR 46–48	Cytotoxic	Izumikawa et al. (2010b)
Halichondria panicea	*Nocardiopsis* strain HB383	Nocapyrones A–D	Cytotoxicity, antimicrobial	Schneemann et al. (2010a)
Halichondria panicea	*Streptomyces* sp. strain HB202	Mayamycin	Cytotoxicity, antibacterial	Schneemann et al. (2010b)
Haliclona sp.	*Streptomyces* sp. Sp080513GE-	Fumaquinone (isoprenoids)	Cytotoxic activity	Khan et al. (2010)
Unidentified sponge	*Actinomadura* sp.	JBIR-65	Radical scavenging activity	Takagi et al. (2010)
Cinachyra sp.	*Micromonospora* sp.	Squalene (isoprenoids)	Cytotoxic activity	Khan et al. (2010)
Cinachyrella australiensis	*Streptomyces* sp. DA18	Cyclo-(L-Pro-D-Phe), cyclo-(D-Pro-D-Phe), cyclo-(D-6-Hyp-L-Phe), and cyclo-(L-Pro-D-Val) (diketopiperazines, cyclic peptides)	Antimicrobial activity	Gao et al. (2010)
Demospongiae sponge	*Streptomyces* sp.	Salicylamide derivative, JBIR-58	Cytotoxic activity	Ueda et al. (2010)
Haliclona sp.	*Streptomyces* sp. Sp080513GE-26	Tetracenoquinocin and 5-iminoaranciamycin	Cytotoxic activity	Motohashi et al. (2010a)
Callyspongia sp.	*Brevibacterium* sp. KMD 003	6-Hydroxymethyl-1-phenazine-carboxamide and 1,6-phenazinedimethanol	Antibacterial	Choi et al. (2009)
Marine sponge	*Saccharopolyspora taberi* PEM-	Angucyclinone	Antitumor	Perez et al. (2009)
Halichondria panicea	*Streptomyces* sp. HB202	Streptophenazines A–H (phenazines)	Antibacterial activity	Mitova et al. (2008)

Pseudoceratina clavata	*Salinispora* sp. strain M403	Rifamycins B and SV	Antibacterial	Kim et al. (2006)
Sponge sp.	*Streptomyces* sp. strain M1087	Nocardamine, dehydroxy, desmethylenyl	Inhibition of enzyme sortase B	Lee et al. (2005)
Mycale plumose	*Saccharopolyspora* sp. nov.	Metacycloprodigiosin and undecylprodigiosin	Cytotoxic; antiplasmodial	Liu et al. (2005)
Halichondria panicea	*Microbacterium* sp. strainHP2	1-*O*-acyl-3-[a-glucopyranosyl-(1–3)-(6-*O*-acyl-amannopyranosyl)] glycerol	Antitumor	Lang et al. (2004) Wicke et al. (2000)
Clathrina coriacea	*Micromonospora* sp. L-31	40-*N*-methyl-50-hydroxystaurosporine	Cytotoxic	Hernandez et al. (2000)
Sponge	*Micromonospora* sp. L-25-ES25-	IB-96212	Cytotoxic	Fernandez-Chimeno et al. (2000)
Xestospongia sp.	*Micrococcus luteus*	Acyl-1-(acyl-60-mannobiosyl)-3-glycerol,2,4,48-trichloro-28-hydroxydiphenylether	Antibacterial and antifungal	Bultel-Ponce et al. (1998)
Xestospongia sp.	*Micrococcus luteus*	Lutoside	Antibacterial activity	Bultel-Ponce et al. (1998)
Corals				
Unidentified Soft coral	*Micromonospora* sp. ACM2- 092	Mozambique, thiocoraline	Anticancer activity	Erba et al. (1999)
Mollusks				
Conus rolani	*Nocardiopsis alba* CR167	Nocapyrones A–C and H–Q (pyrone)	Neurological activity	Lin et al. (2013)
Chicoreus nobilis	*Streptomyces* sp. CN48	Nobilamides A–H, related compounds, and *N*-acetyl-L-phenylalanyl-L-leucinamide	Inhibit mouse and human TRPV1 channels	Lin et al. (2011)
Anadara broughtonii	*Saccharothrix espanaensis*	Saccharothrixins A–C, saccharothrixmicines A and B	Antimicrobial activity	Kalinovskaya et al. (2008)
Conus pulicarius	*Streptomyces* sp. CP32	Pulicatins A–E and five known analogs	Neurological activity	Lin et al. (2010a, b)

(continued)

Table 1 (continued)

Sources	Species	Compounds	Activity	Reference
Unidentified mollusk	*Streptomyces* sp. SCRC-A20	Aburatubolactam A	Cytotoxicity, antimicrobial activity, and inhibition of superoxide generation	Henderson and Phillips (2008)
Ascidians				
Aplidium lenticulum	*Streptomyces* sp. JP90	Cinnamoylphosphoramide	Inhibition of acetylcholinesterase	Quitschau et al. (2008)
Greenish ascidian	*Streptomyces* sp. YM14-060	Piericidins C7 and C8 (polyketide)	Neurological activity	Hayakawa et al. (2007a, b)
Tunicate				
Unidentified tunicate	*Streptomyces* sp. BOSC-022A	Barmumycin	Cytotoxic activity	Lorente et al. (2010)
Marine invertebrate	*Streptomyces* sp. BL-49-58-005	3,6-Disubstituted indoles	Cytotoxic	Lopez et al. (2003)
Seaweed				
Analipus japonicus (Harvey) Wynne	*Streptomyces* sp. YM5-799	Streptobactin, dibenarthin, tribenarthin, and benarthin	Fe-chelating activity	Matsuo et al. (2011)
Seagrass				
Syringodium isoetifolium	*Streptomyces* sp. RAUACT-1	1,4-Dihydroxy-2-(3-hydroxybutyl)-9, 10-anthraquinone 9,10-anthrac	Antibacterial	Ravikumar et al. (2012)
Mangroves				
Bruguiera gymnorrhiza	*Streptomyces* sp. HKI0576	Divergolides A–D (Ansa macrolides)	Cytotoxic and antibacterial	Ding et al. (2011a)
	Streptomyces sp. HKI0595	Xiamycins A and B, indosespene, and sespenine (indolosesquiterpenes)	Antimicrobial activity	Ding et al. (2011b)
	Streptomyces sp. GT2002/1503	Xiamycin and its methyl ester	Anti-HIV activity	Ding et al. (2010)
Aegiceras corniculatum	*Nocardiopsis* sp. A00203	Norcardiatones A–C	Cytotoxic activity	Lin et al. (2010a, b)

44 genera of the sponge-derived actinobacteria have been identified based on culture-dependent methods (Zhang et al. 2008; Jiang et al. 2008; Sun et al. 2010; Khan et al. 2012). Phylogenetic classification using RDP classifier indicated that the sponge-associated actinobacteria consisted of three orders, namely, *Acidimicrobiales*, *Actinomycetales*, and Rubrobacteriales, which together encompassed 112 genera in 39 families. Eighty-seven unique *Streptomyces* were obtained from homogenized gut materials of marine ornamental fish, and these *Streptomyces* species effectively produced bioactive metabolites against target pathogens (Sheeja et al. 2011). Subsequently, Sanchez et al. (2012) have reported three suborders of actinobacteria (*Corynebacterineae*, *Micromonosporineae*, and *Micrococcineae*) from the fish microbiome.

3 Biomedical Application of Compounds Isolated from Marine Actinobacteria

Microorganisms existing in marine environment are having enormous genetic diversity look likely to become a rich source of novel effective drugs. Marine bacteria represent dramatically different environment than their terrestrial counterpart (Parkes et al. 1994). These bacteria originally developed in sediments but also spread in open oceans and allied with the marine organisms. Bioactive compounds documented from marine invertebrates are derived by their associated microbes. Race among microbes for space and nutrients in the marine is a driving energy behind the production of such prized antibiotics and other pharmaceuticals. Fascinatingly microorganisms associated with marine invertebrates and vertebrates are demonstrated as respected candidates for drug unearthing program (Hentschel et al. 2003; Imada 2004; Thakur et al. 2005). At present, 60% of the drugs available in the market were resultant from natural bioproducts (Newman and Cragg 2007). Microorganisms associated with the marine living creatures have the ability to yield diverse and novel natural products (Konig et al. 2006; Piel 2004). Especially actinobacteria are well adapted to live with marine organisms such as sponges, corals, fish, crab, sea cucumber, mollusks, ascidians, seaweeds, seagrasses, and mangroves in the marine environment (Webster and Taylor 2012; Wu et al. 2012; Jimenez et al. 2013; Li et al. 2013; Yang et al. 2013; Abdelmohsen et al. 2014) and are the most important producers for natural products with pharmacological applications (Konig et al. 2006; Dharmaraj and Sumantha 2009; Abdelmohsen et al. 2010; Zotchev 2012).

Natural products derived from the marine organism-associated actinobacteria, about 58% of bioproducts are derived from the marine sponge-associated actinobacteria, while the remaining are from the actinobacteria associated with mollusks, mangroves, corals, ascidians, and seaweeds (Abdelmohsen et al. 2014; Karuppiah et al. 2014). All these natural products were isolated from the genera belonging to *Actinomycetales*. Among them, *Streptomyces* accounts for the largest number of

natural products (66%), followed by *Micromonospora* and *Nocardiopsis* (7%). A percentage of natural product production from the rare actinobacteria like *Actinomadura*, *Salinispora*, *Microbacterium*, *Micrococcus*, *Saccharothrix*, *Saccharopolyspora*, and *Verrucosispora* was about 5, 5, 2, 2, 2, 2, and 2%, respectively (Karuppiah et al. 2014). Between the diverse microbial phyla in marine environment, actinomycetes produced the major fraction of bioproducts (Newman and Hill 2006), with bioactivities counting antibacterial, antifungal, antiparasitic, antimalarial, immunomodulatory, anti-inflammatory, antioxidant, anti-Alzheimer, analgesic, antiarthritic, antifouling, and anticancer activities (Bull and Stach 2007; Baskaran et al. 2016) (Table 1). Therefore, marine actinobacteria especially marine flora and fauna-associated actinobacteria signify an imperative resource for marine drugs (Manivasagam et al. 2013; Abdelmohsen et al. 2014; Karuppiah et al. 2014).

Schematic diagram of isolation, purification, and identification bioactive compound is given in Fig. 1. Depending on the chemical structure, the natural products are classified into polyketides, peptides, isoprenoids, phenazines, alkaloids, fatty acids, indolocarbazoles, plasticizer, polyketides, terpenes, and sterols (Abdelmohsen et al. 2012; Subramani and Aalbersberg 2012; Tiwari and Gupta 2012; Blunt et al. 2013; Baskaran et al. 2016).

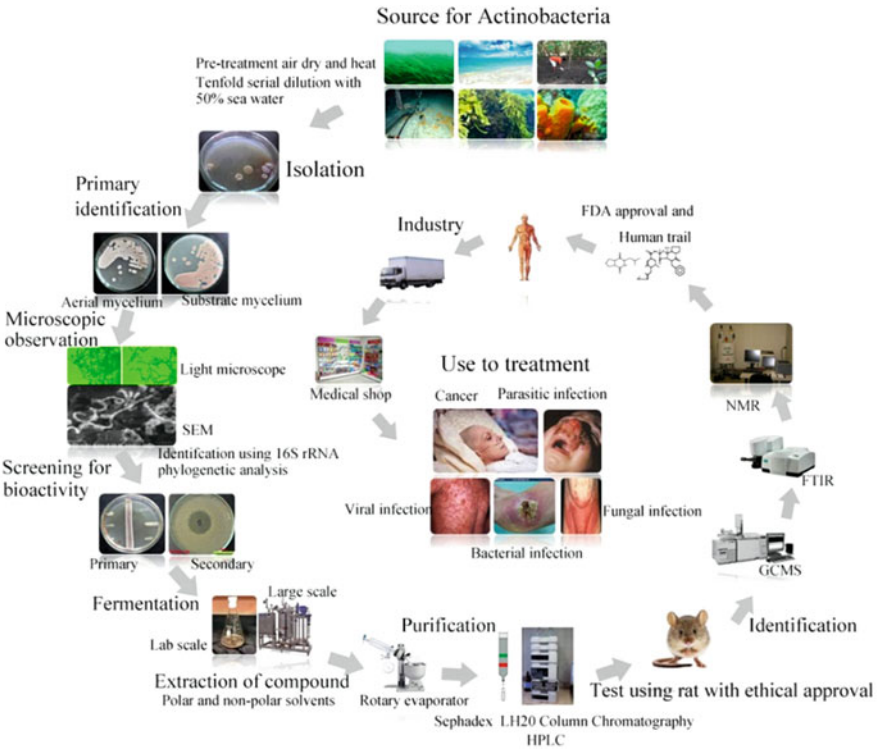

Fig. 1 Schematic diagram of isolation and identification of bioactive compounds from marine actinobacteria

Bioactive compounds, activity, source, and actinobacteria associated with marine living creature are given in Table 1. Some compound activity against cancer, pathogenic bacterial and fungal, and other human diseases has been discussed.

3.1 Cancer

Two staurosporine derivatives, 40-*N*-methyl-50-hydroxystaurosporine and 50-hydroxystaurosporine isolated from *Micromonospora* sp. L-31drived from marine sponge, have potential cytotoxic activities against human lung adenocarcinoma, colon adenocarcinoma HT-29, and melanoma SKMEL-28 (Fernandez-Chimeno et al. 2000). Liu et al. (2005) reported that metacycloprodigiosin and undecylprodigiosin have significant activity against human peripheral blood promyeloblast HL-60, lung carcinoma A-549 and SPCA4, and hepatic carcinoma BEL-7402, which was isolated from *Saccharopolyspora* sp. associated with marine sponge (*Clathrina coriacea*). Likewise Perez et al. (2009) have also reported a potential compound (angucyclinone) against human breast adenocarcinoma MDA-MB-231, human colorectal adenocarcinoma HT-29, and human lung carcinoma A-549 cells, which was extracted from *Saccharopolyspora taberi* isolated from marine sponge. Arenicolide compounds exhibited antitumor activity; arenicolide A showed medium cytotoxicity against the human colon adenocarcinoma cell line HCT-116 with an IC50 value of 30 μg/m (Williams et al. 2007).

Aranciamycin isolated from a sponge-derived *Streptomyces* sp. is reported for its potential cytotoxicity against human cervical carcinoma HeLa cells and human acute myelogenous leukemia LH-60 cells. Similarly, a new teleocidin analog JBIR-31 has also been reported for cytotoxic activity against cervical carcinoma HeLa cells (Izumikawa et al. 2010a). Asolkar et al. (2006) reported that daryamides A, B, and C extracted from *Streptomyces* sp. derived from marine sediment have potential activity against human colon carcinoma cell line HCT-116.

The compound GGL.2 1-*O*-acyl-3-[a-glucopyranosyl-(1–3)-(6-*O*-acyl-amannopyranosyl) isolated from *Microbacterium* sp. strain HP2 associated with *Halichondria panicea* was reported to prevent growth of the tumor cell lines HM02 and Hep G2 (Lang et al. 2004). Two novel kijanimicin by-products, lobophorins D and C obtained from *Streptomyces carnosus* associated with sponge, are recorded for its potential activity against the human liver and breast cancer (Wei et al. 2011). The potent cytotoxic thiodepsipeptide thiocoraline and five new analogs of thiocoraline 2,2′-deoxythiocoraline, thiochondrilline C, and 1,2′-sulfoxythiocoraline exhibited significant cytotoxicity in contradiction of the A-549 human cancer cell line (Wyche et al. 2011). Likewise, several researchers have also reported numerous potential compounds from actinobacteria derived from marine plants, animals, and sediments are given in Table 1. About 39% of compounds isolated from different genera of marine actinobacteria have potential activity against various cancer cell (Fig. 3).

3.2 Antibacterial and Antifungal

The known synthetic 2,4,4,8-trichloro-28-hydroxydiphenylether obtained from *Micrococcus luteus* derived from marine sponge has very strong activity against *Staphylococcus aureus*, *Vibrio anguillarum*, and *Candida albicans* (Bultel-Ponce et al. 1998). Imamura et al. (1993) have also reported that two new antimycins, urauchimycins A and B produced by marine actinobacteria, have potential antifungal activity against *Candida albicans* at an absorption of 10 mg ml/1. Similarly, Kim et al. (2006) have reported that rifamycins are a group of antibiotics with promised activities against Gram-positive bacteria which was produced by *Streptomyces* sp. obtained from marine sponge, *Pseudoceratina clavata*. Rifamycins B and SV were first forecasted by ketosynthase gene sequences of *Salinispora* M403; it showed that the polyketide synthase gene sequence is most closely correlated to that of the rifamycin B synthase of *Amycolatopsis mediterranei*.

Choi et al. (2009) have also reported that actinobacteria (*Brevibacterium* sp.) isolated from marine sponge (*Callyspongia* sp.) have produced potentially active compounds (6-hydroxymethyl-1-phenazine-carboxamide and 1,6-phenazinedimethanol) against *Enterococcus hirae* and *Micrococcus luteus* growth was suppressed. Also mayamycin obtained from *Streptomyces* sp. associated with *Halichondria panicea* has potential activity against *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) with IC50 values of 0.31 to 31.2 μM (Schneemann et al. 2010a, b). Likewise, abyssomicin C extracted from *Verrucosispora* sp. derived from marine sediments is highly active against vancomycin-resistant *Staphylococcus aureus* (Bister et al. 2004). Similarly, Gao et al. (2012) have documented that *N*-(2 hydroxyphenyl)-2-phenazinamine (NHP) purified from *Nocardia dassonvillei* obtained from marine sediments has potential antifungal activity.

Two new anthraquinone (C-glycosides: himalomycins A and B) antibiotics with the infrequent fridamycin E chromophore, an ancestor of the anthracycline antibiotics, have been reported from actinobacteria. Himalomycins were isolated from marine sediment actinobacteria and have been recorded for its strong activity against *B. subtilis*, *S. viridochromogenes*, *S. aureus*, and *E. coli* (Maskey et al. 2003). Macrolides are protein synthesis inhibitors. *Bacillus subtilis*, *E. coli*, and *S. aureus* were maximum sensitive against chalcomycin A; however, *S. viridochromogenes* showed very weak (Wu et al. 2007). Bonactin is a novel antimicrobial chemical compound, which is obtained from marine sediment *Streptomyces* sp. BD21 was recorded for its potential activity against Gram-positive and Gram-negative bacteria and antifungal activity (Schumacher et al. 2003).

3.3 Antimalaria, Anti-inflammatory, Antifouling, etc.

Trioxacarcins A, B, and C, A purified from marine soil *Streptomyces ochraceus* and *Streptomyces bottropensis*, were recorded for its highest antiplasmodial activity,

which is similar to that revealed by artemisinin (Maskey et al. 2004). As well as butenolides isolated from marine actinobacteria has potential activity against fouling, estrogenic, serotonergic, and HIV (Husain et al. 2010; Michel et al. 2010; Shi et al. 2012). Pimentel-Elardo et al. (2011) have also recorded strong inhibitory activity of tetromycins against cysteine proteases and malaria, which was derived from marine sponge (*Axinella polypoides*)-associated *Streptomyces axinellae*. Also, Isaka et al. (2002) found a potential activity of metacycloprodigiosin against *Plasmodium falciparum* K1 obtained from marine sponge-associated *Saccharopolyspora* sp. nov. Staurosporine and valinomycin were reported for its noteworthy antiparasitic activity against *Leishmania major* and *Trypanosoma brucei brucei*, which was isolated from *Streptomyces* derived from marine sponges (Pimentel-Elardo et al. 2010).

Abdelmohsen et al. (2014) have reported antioxidant activity of diazepinomicin by both chemical and cellular methods against human kidney (HK-2) and human promyelocytic (HL-60) cell lines, which was obtained from *Micromonospora* sp. associated with marine sponge. Moreover, diazepinomicin suppressed the proteases rhodesain and cathepsin Lat an IC50 of 70–90 μM, as well as exhibited antiparasitic activity against trypomastigote forms of *Trypanosoma brucei* with an IC50 of 13.5 μM (Abdelmohsen et al. 2012). Likewise, manzamines isolated from marine actinobacteria have been reported as potential compounds to inhibit the malaria, tuberculosis, and HIV agents (Radwan et al. 2012). Salinamides A and B isolated from marine actinobacteria have been recorded as potential antibiotic and anti-inflammatory agents (Moore et al. 1999). A significant anti-inflammatory activity of cyclomarin A purified from marine actinobacteria was documented in both in vivo and in vitro assays (Renner et al. 1999). A maximum of pharmaceutical compounds obtained from actinobacteria, isolated from various sources of marine, is given in Table 1.

4 Conclusion and Future Perspectives

Actinobacteria are extensively dispersed in different marine environment and have a marvelous ability to deliver bioactive compounds with strong biological activities. Literature survey revealed that actinobacteria and in specific the genus *Streptomyces* have the potential to yield a wide variety potential pharmaceutical compounds, having antimicrobial, anticancer, anti-inflammatory, antimalarial, antiviral, and anti-angiogenesis drugs (Manivasagan et al. 2013; Abdelmohsen et al. 2014; Karuppiah et al. 2014; Baskaran et al. 2016). Bioactive compounds produced actinobacteria in marine environment;~ 52% were isolated from marine sediment and sand followed by marine flora and fauna-associated actinobacteria; among the associated actinobacteria,~ 37% were reported from sponge followed by other flora and fauna (Fig. 2). Bioactive compounds obtained and identified from marine actinobacteria have tremendous application to prevent the human as well as animal disease, and it showed that about 39% compounds represent activity against

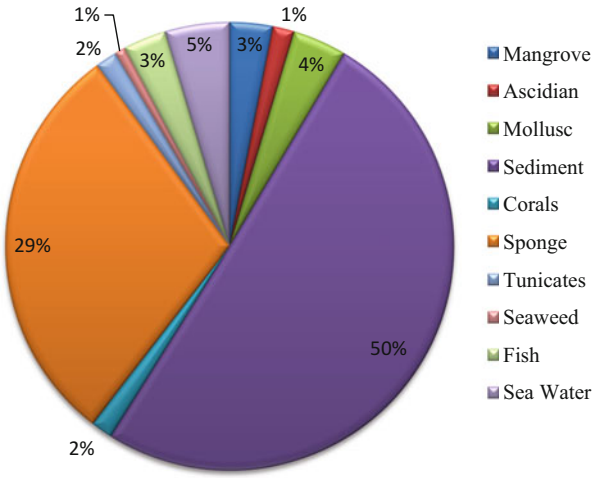

Fig. 2 Major marine source of bioactive compounds produced by actinobacteria

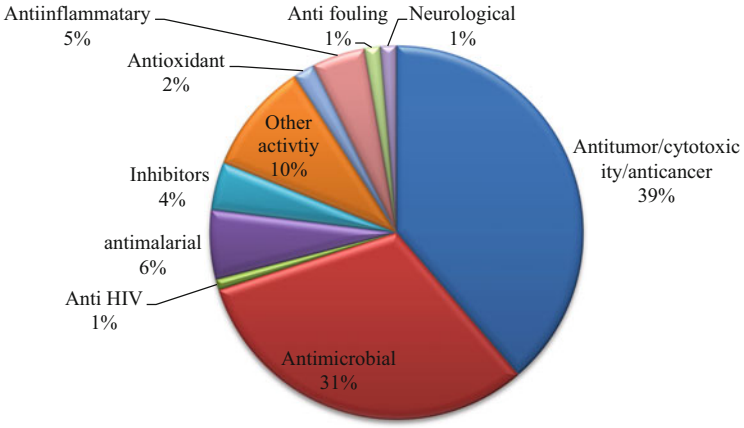

Fig. 3 Pharmaceutical applications of compounds produced by actinobacteria

cancer/tumor/cytotoxicity followed by antimicrobial activity of 31% (Fig. 3). Large numbers of new compounds of pharmacological importance have been reported from the marine actinomycetes especially associated with the living creatures. Many researchers have recapped our trust that bioproduct search and unearthing in marine actinobacteria displays exceptional potential in pharmaceutical uses. Marine actinomycetes have established for its important genomic and metabolic assortment; however, efforts should be focused to discover the new bioactive molecules. Since the majority of the microbes associated with the marine organisms are not cultured in the laboratory, hence there must be a big challenge to use the genetic resources of uncultured actinobacteria for producing natural products.

Metagenomics-based soundings, especially the cloning of gene cluster and its heterologous expression, exemplify an important task for natural products production from actinobacteria in marine environment especially associated with marine organisms (Fortman and Sherman 2005; Hentschel et al. 2012).

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Antimycobacterial Agents: To Target or Not to Target

Andaleeb Sajid, Gunjan Arora, Richa Virmani, and Anshika Singhal

Abstract *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium ulcerans* are the three most common pathogens of mycobacterium genus. *M. tuberculosis*, the causative agent of tuberculosis in humans, is the most dangerous bacterial pathogen. In fact as per latest WHO report, *M. tuberculosis* killed more people than HIV in the last few years and is now the leading cause of death. The worst affected from global TB epidemic are nine high-burden countries which includes China and India. The 23% of total TB cases are reported from India, indicating that more serious efforts are needed to tackle the TB incidence, prevalence, and mortality. Another concern is rise of multiple drug-resistant *M. tuberculosis* strains and all current antimycobacterial agents will no longer be effective in the future. Research in TB drug discovery remains abysmal and there is shortfall of USD 1.6 billion to treat the most neglected disease. TB does not attract major funding from developed countries which prioritize cancer, HIV, and malaria. As drug discovery is getting costlier, most of the major pharmaceutical companies do not find TB research financially viable. This has resulted in major gap between drug discovery pipeline and need for new drugs. The challenge is to stop global TB epidemic by finding new antimycobacterial agents that effectively treat MDR-TB and XDR-TB in few months and not years. This chapter will describe current antimycobacterial agents, their mechanisms, and new candidate molecules in clinical trials. The goal is to understand how the old drugs worked and how we can design new strategies to develop new antimycobacterial agents.

Keywords Mycobacterium tuberculosis • Tuberculosis • DOTS • Drug targets • Drug-sensitive TB • MDR TB • XDR TB • Drug resistance

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1 Introduction

TB is a highly contagious disease which is caused by *Mycobacterium tuberculosis*, a pathogenic bacterium that spreads through aerosol. *M. tuberculosis* is one of the greatest causes of deaths by a pathogen worldwide and about 1/3rd of the world population harbors this pathogen. The major symptoms of TB include severe cough, pain in chest, loss of weight, high fever, and bloody mucus. It is stated that not all TB infections lead to development of disease and the infection can be in latent stage for most part (Barry et al. 2009; Dheda et al. 2015; Fogel 2015; Getahun et al. 2015). During infection, *M. tuberculosis* resides within lung alveolar macrophages, where it can be in non-replicating persistent (NRP) form or may be actively dividing (Russell 2001; Mwandumba et al. 2004). The NRP form may start replicating actively when the immune system is compromised due to one or more reasons, for example, damage from AIDS or diabetes, drug abuse, chemotherapy, old age, or other causes, leading to development of active TB disease (Stead and Dutt 1989; Kamboj and Sepkowitz 2006; Martinez and Kornfeld 2014; Wasserman and Meintjes 2014; Sullivan et al. 2015). The success of *M. tuberculosis* as a pathogen lies in its ability to reside within the macrophages that are considered the pillars of immune system. Once the immune system is compromised, it is almost impossible to overcome mycobacterial infection that can be fatal if not treated by drugs.

2 Existing Treatment Regime

2.1 Drug-Sensitive TB

Since its revelation, there have been unlimited attempts to circumvent TB infection. Right from the discovery of streptomycin in 1943 as a first successful anti-TB drug, there have been many more drug candidates (Sotgiu et al. 2015). Resistance from streptomycin arose within few years and, hence, the need for new drugs. Another compound called para-aminosalicylic acid (PAS) was being used along with streptomycin to slow down the generation of resistance, but was required to be taken for longer periods of time (Schweinle 1990). Within 10 years of this regimen, isoniazid was discovered as potent antimycobacterial agent. These three drugs were able to completely cure the TB disease, provided taken for sufficient period of time. But due to lack of constant supervision, most patients did not comply with the complete regimen and stop taking medicines (Mitchison 1998). These practices lead to development of resistant forms of bacteria that were still remaining in the patients. Following this, the agencies prescribed the "direct observed treatment, DOT" program for treatment of TB patients (Ogden et al. 1999; Karumbi and Garner 2015). Later on, rifampicin, pyrazinamide, and ethambutol were added to the treatment along with streptomycin and isoniazid that lead to further reduction of

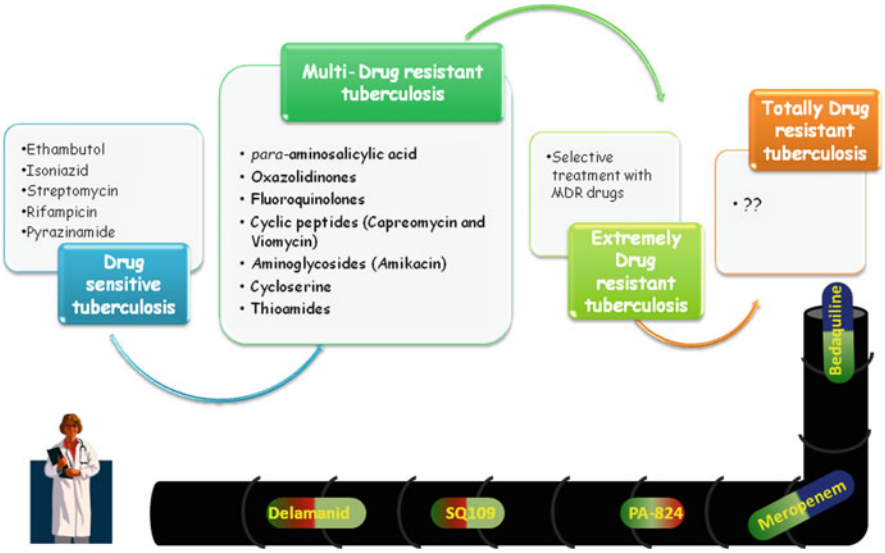

Fig. 1 Tuberculosis treatment regime: Schematic diagram showing the drugs used for different forms of tuberculosis as first-line and second-line tuberculosis. The diagram also shows the most effective drugs in the pipeline to be approved for tuberculosis treatment

relapsed rate, and treatment was shortened to 6 months (famous as DOTS program, directly observed treatment, short course). These drugs were considered the first-line treatment for TB infections worldwide (Sterling et al. 2011; Tripathi et al. 2012; Sotgiu et al. 2015) (Fig. 1, Table 1).

In many developing countries, due to expense of treatment and toxic effects, the TB treatment was limited to isoniazid alone (Ogden et al. 1999). This led to development of isoniazid-resistant strains of *M. tuberculosis*. Subsequently, due to inability to reach effective drug concentration for treatment, most of the *M. tuberculosis* strains became resistant to one or more existing drugs, a phenomenon supported by continuous mutations in *M. tuberculosis* strains. These strains were considered multidrug-resistant (MDR) TB (Aziz and Wright 2005; Harries and Dye 2006; Sharma and Mohan 2006).

2.2 Multidrug-Resistant (MDR) TB

Resistance in *M. tuberculosis* strains has been encountered against one or more first-line drugs. MDR-TB strains are defined as being resistant to treatment with isoniazid or rifampicin, the two most potent first-line drugs (WHO 2009; Telenti et al. 1993; Dalton et al. 2012; Fogel 2015). The current worldwide status of TB infection claims that a major proportion of the *M. tuberculosis* strains are now MDR (http://www.who.int/tb/post2015_strategy/en/). Clinically, it is rather difficult to

Table 1 Antimycobacterial agents: treatment modules and mechanism of action

Drug	Treatment	MIC against *M. tuberculosis*	Mechanism of action	Bacterial spectrum	Reference(s)
First-line drugs					
Isoniazid	Oral dose	0.01–0.02 μg/ml	Blocks cell wall mycolic acid	Narrow	Lei et al. (2000)
Ethambutol	Oral dose	5–6 μg/ml	Obstructs the formation of cell wall	Narrow	Jadaun et al. (2007)
Streptomycin	Oral dose	1–2 μg/ml	Inhibits protein synthesis	Broad	Heifets and Lindholm-Levy (1989)
Rifampicin	Oral dose	0.02–0.125 μg/ml	Inhibits transcription by targeting RNA polymerase	Broad	van Den et al. (2009)
Pyrazinamide	Oral dose	50–100 μg/μl	Inhibits membrane transport function	Narrow	Zhang et al. (2003)
Second-line drugs					
Para-aminosalicylic acid	Oral dose	5 μg/ml	Targets folate pathway	Broad	Saifullah et al. (2014), Zheng et al. (2013)
Oxazolidinones	Oral dose	0.125–1 μg/ml	Inhibits protein synthesis	Broad	Williams et al. (2009), Zhang et al. (2014)
Cyclic peptides (capreomycin and viomycin)	Intravenous/ intramuscular	2.5 μg/ml	Inhibits protein synthesis	Broad	Khardori et al. (1994)
Aminoglycosides (amikacin)	Intravenous/ intramuscular	4–8 μg/ml	Targets 30S ribosome	Broad	Heifets and Lindholm-Levy (1989)
Cycloserine	Oral	32 μg/ml	Acts as a peptidoglycan inhibitor	Broad	Lambert and Neuhaus (1972), Rastogi and David (1993)
Fluoroquinolones	Oral/ parenteral	0.25–0.50 μg/ml	Inhibits bacterial DNA gyrase	Broad	Rodriguez et al. (2001)
Thioamides	Oral	0.5 μg/ml	Disrupts mycolic acid	Narrow	Wang et al. (2007)
Bedaquiline	Oral	0.65 μg/ml	Inhibition of F0-F1 ATP synthase	Narrow	Keller et al. (2015)

Drugs under clinical trials					
PA-824	Oral	0.015–0.25 μg/ml	Affects protein and lipid synthesis	Broad	Lenaerts et al. (2005), Singh et al. (2008), Stover et al. (2000)
SQ-109	Oral	0.12–0.36 μg/ml	Affects cell wall synthesis by targeting MmpL3	Broad	Protopopova et al. (2005), Tahlan et al. (2012)
Meropenem	Intravenous	0.125 and 2 μg/ml	Inhibits bacterial cell wall synthesis	Broad	Davies et al. (2015), Kumar et al. (2012)
Sutezolid	Oral	0.05 μg/ml	Binds to 23s ribosome and inhibits protein synthesis	Broad	Zhu et al. (2014)
Delamanid	Oral	10 ng/ml	Mycolic acid synthesis	Narrow	Esposito et al. (2015), Keller et al. (2015), Lewis and Sloan (2015), Patel et al. (2015)
AZD5847	Oral	1 μg/ml	AstraZeneca oxazolidinone targets protein synthesis machinery	*M. tuberculosis*, not defined for other bacteria	Balasubramanian et al. (2014a, b), Coleman et al. (2014)
Rifapentine	Oral	0.125 μg/ml	Rifampicin analog	*M. tuberculosis*, not defined for other bacteria	Bemer-Melchior et al. (2000), Sharma et al. (2013)

prove whether the MDR-infected patient developed resistance or was infected by MDR strain, which makes it difficult to choose the right treatment. For these reasons, it is important to find new antitubercular agents with faster bactericidal effect and low frequency of resistance. Unfortunately, most of such compounds have strong toxicity to host tissues associated with them. There are many new drugs with varying specificities and toxicities that are used to treat MDR-TB as second-line drugs (Fig. 1, Table 1). These include the drugs belonging to different groups of chemical compounds—oxazolidinones, diarylquinolines, fluoroquinolones, nitroimidazopyrans, ethylenediamines, and benzothiazinones (Field 2015; Thee et al. 2015) (Table 1, http://www.tbfacts.org/tb-drugs/). Bedaquiline has been recently approved to be used for treatment of MDR-TB, though the success rate for this drug is debatable (Patel et al. 2014; van Heeswijk et al. 2014; Field 2015). Most of these drugs are meant to be given in combination, for being more effective, and avoid generation of resistance to any particular drug. Although very effective, most of these compounds have shown marked toxicity to human organs, especially because of the need to be taken for longer periods of time. Many patients are not able to comply to complete treatment due to gastrointestinal problems, hepatotoxicity, and surplus neurological disorders (Macgregor and Somner 1954; Das et al. 1973; De and De 2001; French 2003; Owens and Ambrose 2005). As a result of this, the *M. tuberculosis* bacterium is developing resistance to second-line drugs also, leading to generation of extremely drug-resistant (XDR) and totally drug-resistant (TDR) strains.

2.3 *Extremely Drug-Resistant (XDR) and Totally Drug-Resistant (TDR) TB*

The form of MDR-TB disease which is not treatable by two first-line drugs—isoniazid and rifampicin in addition to one of the second-line drugs and any fluoroquinolones—is designated as XDR-TB (Miotto et al. 2015). The threat of XDR-TB is more pronounced in developing and underdeveloped countries that are highly populated and have limited resources for treatment (Chakraborty et al. 2010; Paige and Bishai 2010; Bateman 2015; Li et al. 2015; Schito et al. 2015a; Von Delft et al. 2015). As expected, the treatment of XDR-TB patients is extremely difficult, if possible at all, and the success rate is too low. In most XDR cases, the treatment is not highly selective and many drugs are being tried in combinations, to find effective as well as personalized treatment for a given patient. Undoubtedly, such treatments are difficult, highly expensive, and time taking with limited availability of required drugs in sufficient amounts.

In 2007, a new form of TB was reported that was resistant to treatment with all the first-line and second-line drugs. This was named as totally drug-resistant (TDR) TB (Velayati et al. 2009; Udwadia et al. 2012). TDR-TB represented a diagnostic limitation to find the drug which is active against that particular strain of

M. tuberculosis (Behera 2012; Sullivan and Ben 2013). Due to lack of identification of such drug in stipulated time and eventual death of all the infected patients, TDR-TB is considered as untreatable (Mitchison and Davies 2012; Klopper et al. 2013; Velayati et al. 2013).

3 Mechanism of Action

3.1 First-Line Drugs

3.1.1 Isoniazid

Isoniazid inhibits mycobacterial cell wall synthesis (Bernardes-Genisson et al. 2013; Vilcheze and Jacobs 2014) (Fig. 2). It acts in a prodrug mechanism where it is cleaved and activated by catalase/peroxidase action of KatG (Lei et al. 2000; Slayden and Barry 2000; Vilcheze and Jacobs 2007). This cleavage leads to its binding with NADH to form a complex isonicotinic acyl-NADH that conjugates with enoyl-acyl carrier protein reductase InhA. This conjugation inhibits enoyl-AcpM and fatty acid synthase, leading to inhibition of mycolic acid synthesis, which is specific to *M. tuberculosis* cell wall (Timmins and Deretic 2006) (Table 1).

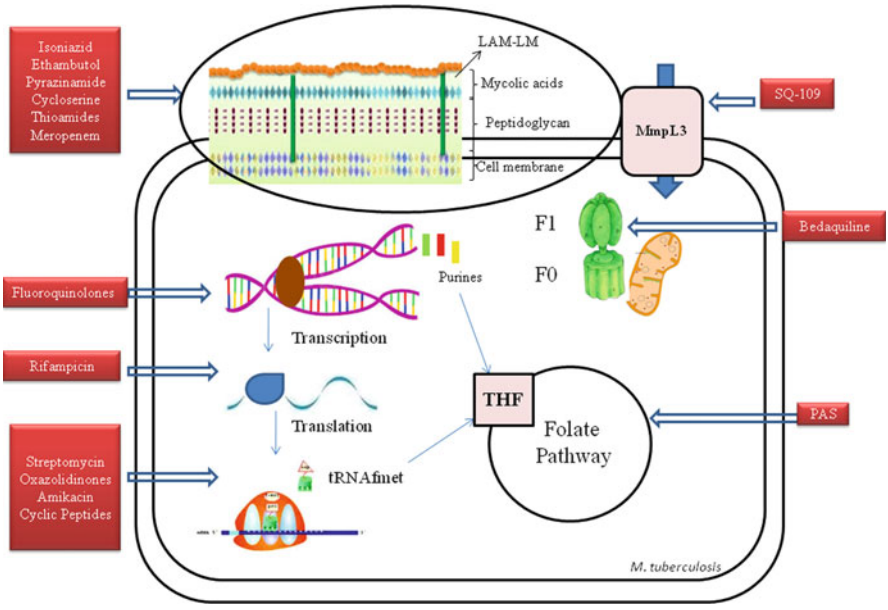

Fig. 2 Antimycobacterial agents and their mechanisms: Tuberculosis drug regimens for a combination therapy are designed on the basis of drugs targeting different pathways essential for *M. tuberculosis* survival. This diagram shows different sets of drugs targeting critical pathways within the cell

3.1.2 Rifampicin

Rifampicin is an inhibitor of mycobacterial transcription machinery (Fig. 2). It blocks the synthesis of RNA from DNA by binding directly to the active site of RNA polymerase enzyme. Thereby, it prevents functioning of RNA polymerase by blocking RNA phosphodiester bond formation (Calvori et al. 1965; Somoskovi et al. 2001; Singh et al. 2006; Tupin et al. 2010).

3.1.3 Ethambutol

Ethambutol is known to modulate the cell wall structure by inhibiting the synthesis of arabinogalactan and its complex with mycolic acid and peptidoglycan (Blanchard 1996; Jankute et al. 2012) (Fig. 2). It is an inhibitor of enzyme arabinosyl transferase, an essential gene in *M. tuberculosis* genome. Ethambutol increases the membrane porosity; hence when given in combination, it helps transport of other drugs inside the *M. tuberculosis* cell (Lee et al. 1997) (Table 1).

3.1.4 Streptomycin

Streptomycin is an aminoglycoside, the first class of antibiotics to be discovered; and it was also the first drug to be approved for treatment of TB. It is a bactericidal compound which targets the basic protein synthesis machinery in bacterial cells (Sharma et al. 2007) (Fig. 2). Streptomycin binds to 16s RNA of 30S ribosomal subunit, leading to blocking of its binding with formyl-methionyl-tRNA (Biswas and Gorini 1972; Demirci et al. 2014). This blocks the protein synthesis at an early step, causing the cell to die. Since eukaryotic protein machinery does not involve similar ribosomes and f-met-tRNA, this drug is specific to bacteria (Table 1).

3.1.5 Pyrazinamide

Pyrazinamide (PZD) follows a prodrug mechanism, which is cleaved inside *M. tuberculosis* to become the active form pyrazinoic acid. Pyrazinoic acid accumulates in the cell under acidic conditions, especially during infection. It is known to target the fatty acid synthesis machinery of *M. tuberculosis* by inhibiting fatty acid synthase I (FAS-I) enzyme (Zimhony et al. 2000, 2007; Zhang et al. 2003) (Fig. 2). Apart from this, there are many off-targets of PZD including the disruption of membrane potential, energy metabolism, and protein synthesis (Zhang and Mitchison 2003).

3.2 Second-Line Drugs

3.2.1 Oxazolidinones (Linezolid)

Oxazolidinones are synthetic antibiotics that act by inhibiting protein synthesis in bacterial cells (Fig. 2). They bind to the P site at the ribosomal 50S subunit and block the formation of its complex with 30S ribosome, tRNA, and mRNA, thus inhibiting the initiation step of protein synthesis (Colca et al. 2003; Bozdogan and Appelbaum 2004). Linezolid was the first oxazolidinone available and has been used for treatment of infections by Gram-positive bacteria. However, there is recent surge of TB treatment by linezolid and has been showing promising results, especially in drug-resistant TB cases (Lee et al. 2015). Currently, the second oxazolidinone, sutezolid, with similar structure as linezolid, is under the clinical trials for treatment of tuberculosis (Lee et al. 2012; Kumar et al. 2015; Patel et al. 2015) (Table 1).

3.2.2 Fluoroquinolones

The fluoroquinolones, used for the tuberculosis treatment as second-line drugs (Ziganshina et al. 2013), mainly act by causing DNA damage and interfering with DNA repair mechanisms (Fig. 2). They have been divided into generations of antibiotics depending on their antibiotic spectrum. The first- and second-generation fluoroquinolones (e.g., nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin) inhibit topoisomerase II ligase domain which eventually leads to DNA fragmentation due to nuclease activity of topoisomerase II. The third and fourth generations of fluoroquinolones (e.g., levofloxacin, gatifloxacin, moxifloxacin) inhibit the ligase domain of topoisomerase IV, which is a conserved enzyme (Hooper 2000; Matrat et al. 2008; Pantel et al. 2011; Maruri et al. 2012; Zhu et al. 2012). Thus, these generations of antibiotics have broad spectrum of targets (Table 1).

3.2.3 Para-aminosalicylic Acid (PAS)

Despite being used as an antimicrobial agent for 60 years, the mechanism of action of PAS has recently been demonstrated (Zheng et al. 2013; Donald and Diacon 2015). In the folic acid synthesis pathway, two enzymes dihydropteroate synthase (DHPS) and dihydrofolate synthase (DHFS) interact with PAS and generate hydroxyl dihydrofolate antimetabolite. This molecule in turn inhibits the essential enzyme dihydrofolate reductase (DHFR) (Rengarajan et al. 2004; Zheng et al. 2013) (Fig. 2, Table 1).

3.2.4 Cyclic Peptides (Capreomycin and Viomycin)

Cyclic peptides that are used to treat MDR-TB are also called as tuberactinomycins. Capreomycin and viomycin are two primary tuberactinomycins. They exert their antibacterial effect by inhibiting the protein synthesis in bacteria (Fig. 2). 23S rRNA and 16S rRNA interact with each other and form a bridge. These cyclic peptides bind to this bridge and inhibit the translocation of peptide during translation elongation step (Johansen et al. 2006; Stanley et al. 2010; Akbergenov et al. 2011).

3.2.5 Aminoglycosides (Amikacin, Kanamycin, Capreomycin)

Amikacin is an injectable anti-TB drug used for treatment of MDR cases (Ristuccia and Cunha 1985; Zaske and Crossley 1978). It is derived from kanamycin and has similar mechanism of action, which is through inhibition of protein synthesis (Alangaden et al. 1998) (Fig. 2). Amikacin interferes with mRNA binding and tRNA acceptor site by primarily binding to 30S ribosomal subunit. This leads to blocking of peptide elongation or production of nonfunctional or even toxic proteins (Jugheli et al. 2009; Georghiou et al. 2012; Sirgel et al. 2012; Du et al. 2013).

3.2.6 Cycloserine

Cycloserine is a pseudo-amino acid which is cyclic analog of D-alanine. Cycloserine targets cell wall synthesis machinery and specifically inhibits peptidoglycan synthesis (Fig. 2). It inhibits two enzymes in the bacterial cell: D-alanine racemase and D-alanyl-D-alanine synthetase causing accumulation of the peptidoglycan precursor UDP-glycolyl muramyl tripeptide (Takayama et al. 1970; Lambert and Neuhaus 1972; Rastogi and David 1993; Prosser and de Carvalho 2013). Due to its severe neurological side effects, the use of cycloserine is restricted for treatment of XDR-TB (Yew et al. 1993).

3.2.7 Thioamides

Ethionamide (ETH) and prothionamide (PTH) are signature thioamides that have been used for TB treatment, specifically for drug-resistant forms. The structure and mechanism of ETH action are similar to that of INH as it disrupts mycolic acid synthesis (Banerjee et al. 1994) (Fig. 2). ETH is a prodrug that is activated by EthA (a monooxygenase) and forms adduct with NAD+ (Vale et al. 2013; Vannelli et al. 2002; Wang et al. 2007).

3.2.8 Bedaquiline

Bedaquiline or TMC207 is the first drug approved specifically for the treatment of drug-resistant TB after 40 years (Diacon et al. 2009; Matteelli et al. 2010). It is a diarylquinoline that targets ATP synthase proton pump and affects ATP synthesis (Balemans et al. 2012; Haagsma et al. 2011) (Fig. 2). The use of bedaquiline has been restricted to the treatment of XDR-TB due to its extreme side effects (Field 2015) (Table 1).

4 Approaches for New Drug Development

There have been continuous efforts for new TB drug development from all parts of the world. Many countries have come up with Global TB Alliance to progress together to fight against this disease (Bridge et al. 2015; Katsuno et al. 2015; Mdluli et al. 2015). With the current rate of resistance development and increasing ineffectiveness of existing drugs, the approaches for new drug development are to be carefully determined (Koul et al. 2011). The principle norms for new drug development are that it should:

1. Target essential component of mycobacterial cell machinery.
2. Have very low MIC against mycobacteria.
3. Be specific for mycobacteria.
4. Be bactericidal.
5. Not be toxic to host/human.
6. Have synergy with other drugs, to be used in combination.
7. Take less time to clear the infection, so that treatment time course can be decreased.
8. Be able to penetrate deeper tissues and granulomas.
9. Be active against non-replicating and dormant mycobacterial cells.
10. Be retained in the body for sufficient time to be able to exert its effects.

These criteria usually define the basis of compound selection and their viability for clinical trials. The new compounds under development need to follow most of these norms, if not all. Many pharmaceutical companies and researchers are developing libraries of chemical compounds to be tested for having anti-mycobacterial activity followed by testing the abovementioned steps.

5 Drugs in Pipeline

With the aim of designing better drugs than the currently existing ones, many new compounds have been developed (Mdluli et al. 2015). Most of these have improved MIC against Mtb with negligible cytotoxicity. These compounds have unique mechanisms of action and usually target an essential component of mycobacterial cell machinery (Table 1). Examples include PA-824, SQ109, meropenem, delamanid, AZD5847, sutezolid, rifapentine, etc. (Fig. 1). Many of these drugs are being used in clinical trials for the treatment of drug-resistant tuberculosis that is not treatable by existing drug regimens and may end up being fatal otherwise (Kwon et al. 2014; Lewis and Sloan 2015; Schito et al. 2015b).

6 Potential Drug Targets

The fact that only a small number of new drugs have been proved successful in TB treatment suggests that efforts are required in this direction. Moreover, besides improving the existing drugs by their chemical modifications, there is a need to identify new proteins and pathways that can be targeted in a combination therapy. Keeping this in mind, and the characteristics of an appropriate target, several new drug targets have been proposed. Most of these targets are essential for bacterial survival (Sassetti et al. 2003) and are involved in a critical function such as proteins involved in metabolic pathways, translation, transport, signaling and sensing, etc. S-adenosylhomocysteine hydrolase (*sahH*) is one such gene that is essential for the survival of *M. tuberculosis*. The enzyme regulates concentration of homocysteine in mycobacteria and may in turn regulate the active methyl cycle (Singhal et al. 2013). Very recently, an essential gene *glmU*, encoding N-acetyl-glucosamine-1-phosphate uridyltransferase, was studied as a candidate drug target (Soni et al. 2015). A novel oxazolidine derivative, Oxa33, has been shown to decrease bacillary load in infected mice by inhibiting GlmU. Another proposed drug target is translational elongation factor Ef-Tu. Antibiotic kirromycin is known to interrupt protein synthesis by stabilizing the GTP-bound form of Ef-Tu (Sajid et al. 2011a). Kirromycin has been shown to differentially inhibit Ef-Tu depending on the phosphorylation status of protein. Thus, protein phosphorylation was proposed as an important regulator while designing new anti-TB molecules.

Several years of research has suggested the essentiality of serine/threonine phosphorylation and tyrosine phosphorylation for bacterial survival and virulence (Pereira et al. 2011; Arora et al. 2012, 2014; Sajid et al. 2015). *M. tuberculosis* encodes 11 Ser/Thr protein kinases, 1 Ser/Thr phosphatase, and 2 Tyr phosphatases. Out of 11 STPKs, PknA, PknB, and PknG are essential for bacterial survival (Sassetti et al. 2003; Koul et al. 2004; Chawla et al. 2014; Nagarajan et al. 2015).

Therefore, these STPKs may be a target of choice for designing new inhibitor molecules. Several chemical libraries are now being tested against these kinases. The molecules targeting STPKs may be optimized for specificity as mycobacterial STPKs group in a distinct cluster than the human kinases. The two tyrosine phosphatases, mPtpA and mPtpB, were found to be dispensable for in vitro growth of bacteria, but were essential for mycobacterial virulence and pathogenesis. Both the phosphatases are secreted by the bacteria in host cytosol. mPtpA helps in blocking phagosome-lysosome fusion by binding to macrophage V-ATPase (Wong et al. 2011). Inhibitor designing against mPtpA has been difficult due to its similarity to human ortholog (Sajid et al. 2015). mPtpB subverts the innate immune response and thus proves to be useful target for designing inhibitor molecules (Zhou et al. 2010; Singhal et al. 2015). Metal ions like zinc have been shown to affect the virulence of *M. tuberculosis* during infection. Zinc has been shown to affect critical proteins that are actually Mn^{2+}/Mg^{2+}-binding proteins such as P(1)-type Mn^{2+}-transporting ATPase and Ser/Thr phosphatase PstP (Botella et al. 2011; Sajid et al. 2011b). Inhibition of phosphatase by zinc is more of conserved phenomena (Sajid et al. 2011b; Arora et al. 2013). These studies indicate that metal ion-based inhibitors can be designed to target a broad spectrum of bacteria. In addition, proteins involved in quorum sensing of bacteria are also considered as unique drug targets as they are highly specific for bacteria (Purohit et al. 2007; Kalia 2013).

Several anti-TB drugs fail during their phenotypic testing due to their export from cells by efflux pumps. *M. tuberculosis* harbors many such pumps, like ATP-binding cassette (ABC) transporters, that are responsible for development of drug resistance. Thus, administering inhibitors of efflux pumps in combination with specific anti-TB molecules should be a method of choice for TB treatment (Botella et al. 2011; Pule et al. 2015). Verapamil, which inhibits ABC transporters, has been shown to decrease the treatment period when administered together with the standard anti-TB drugs.

7 Conclusion

It has been a challenge to understand TB disease and its etiology. Consequently, TB drug discovery is not only complicated but is also laborious, time-taking, and money-consuming process. The efforts toward identifying and marketing new TB drugs have been ever increasing all over the world. The objective is to eradicate TB by 2025 and the progress has been encouraging to achieve this goal.

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Part II
Agriculture

Microbial Biofilm: Role in Crop Productivity

Bhagwan N. Rekadwad and Chandrahasya N. Khobragade

Abstract Bacteria, fungi, and mycorrhizae and their metabolic products when adhere to the biotic and abiotic surfaces as a single or multicellular assembly can be described as biofilms. Beneficial microorganisms associated with plants interact with host tissues and deal with many issues during symbiosis, commensal relationship (mutualism), and pathogenesis. The numbers of beneficial microorganisms associated with plants are less than that present in biofilms and vary from small clusters of cells to extensive biofilms. Beneficial biofilms of bacteria, plant growth-promoting rhizobacteria (PGPR), fungi, and mycorrhizae are the important group of microbial inoculants among the beneficial biofilms forming microorganisms which has been studied extensively for their ability to promote growth of crop plants and their improved ability. PGPR and PGPR-like microorganisms' acts through direct or indirect mechanisms either inhibit or prevent the effects of phytopathogenic microorganisms facilitating the availability and uptake of essential nutrients such as nitrogen, phosphorus, potassium, ferrous, zinc, etc., from the atmosphere (air, water, and soil). Environmental stresses such as high salinity, drought, high or low pH, high or low temperature, pressure of heavy metals like Fe and Ni, flooding, and pathogens have a negative impact on crop plants. These problems can be solved using the PGPR biofilm to enhance the growth and productivity of crops, lower the ethylene concentration, increase phytohormones and exopolysaccharide production, induce systematic resistance in plants, and secrete noticeable quantity of siderophores that helps to solubilize iron (Fe) and increase its availability for plant uptake. Similarly, arbuscular mycorrhizal (AM) biofilm also helps the plant under stressful conditions by regulation of plant nutrition and enhancing production of phytohormones and antioxidant enzymes. Additionally, a variety of siderophores secreted by soil and marine microorganisms made essential minerals available to microbial cells to confer resistance to disease and improved the plant productivity. These polymicrobial, multiple beneficial functions in polyextremophilic condition such as phosphate solubilization, biocontrol, dinitrogen (N_2) fixation performed by biofilms, and consortia of biofilms promote the possible positive new developments

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in this research area. Therefore, it is necessary to develop inoculum of biofilm and consortia of biofilm for increased crop productivity on a large scale.

Keywords AM • PGPR • Dinitrogen • Quorum sensing • Siderophores • Marine bacteria

1 Introduction

A thin coating of living material (living cells and metabolic products) formed by the organisms is known as biofilm. Many organisms produce biofilm, but microscopic biofilms are generally formed by bacteria, fungi, microalgae, and mycorrhizae. Both the Gram-positive and Gram-negative bacteria can form biofilm on abiotic surfaces such as ore (minerals), the dead organisms, and their remainders or at water-air interfaces (Fig. 1). Likely, they have the ability to form biofilm in association with living beings such as plants, animals, and other microbes. Additionally, they also have to secrete sticky extracellular polymeric low molecular weight substances (EPS), which provide structure and protection to the microbial community (O'Gara 2007; Lemon et al. 2008; Senadheera and Cvitkovitch 2008). The mycorrhizal biofilm helps plants in the absorption of nutrient and their recycling and acts as biocontrol agent which fights against pests and diseases. Consequently, these naturally occurring beneficial biofilms improve the density of the soil and improve plant growth.

The biofilms have several beneficial effects such as it induces increased growth of shoots, helps plant in the early formation of leaves, and increases the number of green leaves (Seneviratne et al. 2008; Rekadwad 2014). For biofilm formation and quorum sensing, it is necessary to attain critically high cell density, which may be enhanced by incorporating atmospheric nitrogen fixers to them (Seneviratne 2008;

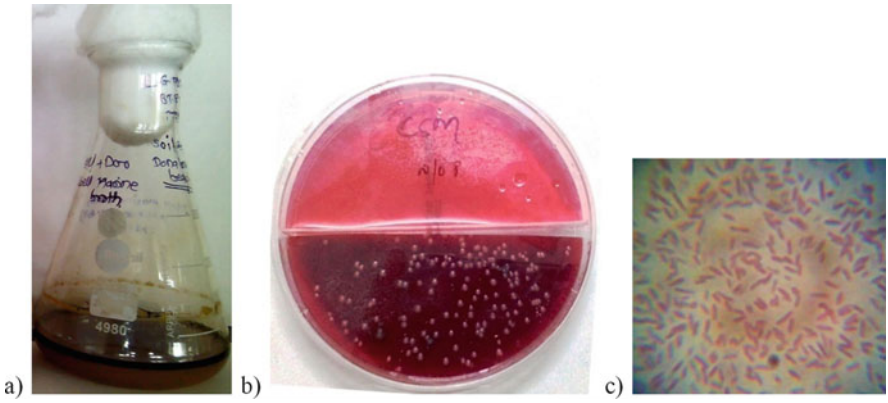

Fig. 1 *B. subtilis*: (**a**) Biofilm in broth, (**b**) bacterial colonies developed on blood-mannitol agar bi-plate, (**c**) microscopic observation

Kalia and Purohit 2011; Kalia 2015). Therefore, it is necessary to develop biofilm in vitro and could be added to agricultural crop to gain increased productivity (Bandara et al. 2006). The microbial biotechnological applications of biofilms have been investigated. In this chapter, the role of microbial biofilm as biofertilizer/bioinoculant, symbiotic and non-symbiotic nitrogen fixers, and association of microbial biofilm with legumes and nonlegumes are discussed, and their different promising applications to increase crop productivity have been explained.

2 Role of Microorganisms in Plant Growth Promotion

Variety of microorganisms acts as biofertilizers. They fix atmospheric nitrogen into soil and made available to plants. This is possible only because of the presence of enzymes such as nitrogenase, cysteine proteases, etc.

2.1 *N_2 Fixation by Root-Associated* Pseudomonas stutzeri *A1501*

Previous studies on *Pseudomonas* genus showed that atmospheric dinitrogen fixation capacity is absent in *Pseudomonas*. This tale showed false by *P. stutzeri* A1501. It is associated with the roots of plants forming a biofilm. Its circular consists of 4,567,418 bp long in size. It has 4146 protein-encoding genes (Rediers et al. 2003; 2007). Its genes are involved in utilization of a wide range of carbon sources (such as glucose, maltose), dinitrogen fixation (because of 59 *nif* genes clustered in the 49 kb nitrogen region PST 1302-PST 1359) (Young et al. 2006), biosynthesis of polyhydroxybutyrate, and multiple pathways for degradation of aromatic hydrocarbons (protocatechuate and catechol) (Lalucat et al. 2006; Pohlmann et al. 2006; Vodovar et al. 2006). Denitrification is another general property of many *Pseudomonas* strains; *P. stutzeri* A1501, *P. stutzeri* Zobell strain, and many other strains have a supercluster of genes (*nor*, *nir*, and *nos)*. The mutants of *nar*G are responsible for coding component of the nitrate reductase enzyme (O'Toole and Kolter 1998; Oueslati 2003; Penrose and Glick 2003; Yan et al. 2008).

2.2 *Plant Growth Promotion by PGPR*

The rhizobacteria such as *Pseudomonas putida*, *Aeromonas*, *Bacillus*, and *Enterobacter* species are collectively named by a single term as PGPR. These PGPR have the inherent ability to colonize effectively in plant roots through direct or indirect mechanisms (Podile and Kishore 2006). Direct mechanism of PGPR includes improved uptake of nutrients such as nitrogen, phosphorus, or production

of phytohormones such as IAA, gibberellins, and cytokinins (Shashidhar and Podile 2010). The PGPR also facilitate development of plants through direct mechanisms. In the direct mechanism, PGPR lowers the ethylene levels by de-amination of 1-aminocyclopropane-1-carboxylic acid (ACC). This reaction is catalyzed by ACC deaminase, which hydrolyzed ACC to α-ketobutyrate and ammonia. Several rhizobacteria have ability to utilize ACC as a sole source of nitrogen (Duca et al. 2014).

Indirect mechanisms principally include microbial antagonism/competitiveness (Kalia 2014) and enhancement of induced systemic resistance (ISR) and suppress the incidence of plant diseases (Vaikuntapu et al. 2014). This can be achieved by producing certain antimicrobials such as antibiotics, hydrogen cyanide, lipopeptide bio-surfactant, and production of siderophores. Some of the PGPR produce hydrolytic enzymes, elicit defense response in plants, and confer resistance against pathogenic bacteria, fungi, and viruses or form protective biofilms on root surfaces (Dutta and Podile 2010; Rekadwad 2015).

2.3 *Role of Fungi in Plant Growth Promotion and Productivity*

Fungi are the heterotrophic mode of nutrition capable to grow on a variety of organic and inorganic (live or dead and/or decaying) substrates. The adhesion of fungal spores or hyphae on the substratum, humidity (60%) and ambient temperature, and pH are the prerequisites for the development of three-dimensional mycelia as a biofilm (Priegnitz et al. 2012). Unlike these, some fungal species can form biofilm under the minimum moisture content, i.e., under low water activity. Moreover, there are some differences in fungal hyphae content and architecture (O'Donnell et al. 2008).

Recent studies showed that biofertilizers reduced time required for seed germination through the vigorous breaking of seed dormancy and balancing the hormonal effects. The higher production of IAA results in slower seed germination. This problem is successfully overcome through regulated production of IAA for increased plant growth using fungal-bacterial biofilms (Marcel et al. 1998, 2006, 2008). It differs from bacterial biofilm and has regulated seed germination and improved plant growth. Fungal-bacterial biofilms encompass one *Aspergillus* species and two or more dinitrogen-fixing bacteria, such as *Azotobacter* sp., *Azospirillum* sp., *Rhizobium* sp., Gram-positive rod, and Gram-negative rod. These extensively tested fungal-bacterial biofilms also warrant becoming good biofertilizers in the biofilm mode for futuristic development agriculture (Buddhika et al. 2014).

2.4 Role of Mycorrhizal Biofilms in Crop Productivity

Mycorrhizas are the bizarre microorganism's lives in symbiotic association with plant roots. Mycorrhizal fungi encompass AM and ectomycorrhizae (EM). AM are the most abundant fungi that are cosmopolitan and common in agricultural soils as well as in un-urbanized soils. They infect roots of terrestrial and aquatic plants and live in symbiotic association (Khan and Belik 1995; Christie et al. 2004; Wu and Xia 2006; Benabdellah et al. 2011; Willis et al. 2013; Chen et al. 2014a, b). Subsequently, after infection these fungi penetrate into cortical cells and form arbuscule that serves as an anchor and help in exchange of nutrient and metabolites between AM and host cytoplasm. The AM hyphae also penetrate through soil deeply which helps in the acquisition of more nutrients, minerals, and water from the soil and also contribute to improving soil structure (Rillig and Mummey 2006; Javaid 2009).

An AM fungus plays very important roles in nutrient recycling/mobilization. Their contribution has played vital role in growth and increased productivity through several ways, such as root colonization (Cavagnaro et al. 2006; Nunes et al. 2010), more absorption and translocation of nutrients than by non-mycorrhizal plants (Guo et al. 2010), increasing availability and supply of slowly diffusing phosphate to the plants (Sharda and Koide 2010), and provision of micro- and macro-nutrients such as nitrogen (N), potassium (K), magnesium (Mg), cupric (Cu), and zinc (Zn), particularly in soil where they are present in less soluble forms (Meding and Zasoski 2008; Smith and Read 2008). In Mediterranean soils with high phosphate-fixing capacity, dinitrogen fixation by rhizobia is weak due to limited supply of phosphorous and micronutrients in less amount and less soluble forms. The AM fungi are commonly associated with legume. The improved nutrient uptake has been observed in legumes with AM and *Rhizobium* (Antunes et al. 2006; Guo et al. 2010; Javaid 2010; Tavasolee et al. 2011).

2.5 Mechanisms Adopted by Plant Growth-Promoting Rhizobia and Mycorrhizae for Plants Under Stress

Extremes, i.e., high salinity, drought, high or low pH, high or low temperature, pressure of heavy metals like ferrous (Fe) and nickel (Ni), flooding, and pathogens, have a negative impact on the growth and productivity of the plants. The hormonal imbalance, nutritional imbalance, ion toxicity, desiccation, and increased susceptibility toward pathogenic attacks are the adverse effects of extremes on plants. These consequences resulted in less crop productivity (Nadeem et al. 2010; Ashraf et al. 2013; Reddy and Saravanan 2013). The PGPR enhances the growth and productivity of crops, lowers the ethylene concentration, increases production of phytohormones and exopolysaccharide, induces systematic resistance in plants, and secretes noticeable quantity of siderophores. This increased siderophore secretion solubilizes iron

(Fe) and increases its availability for plant uptake (Glick et al. 2007; Sandhya et al. 2009). The pathways required for above processes that have to be carried out under stressful conditions depend on the extent of AM and plant (host) association as well as the number of soil, environmental, and plant factors (Nadeem et al. 2013).

2.6 Siderophore-Mediated Ion Acquisition by Microorganisms and Their Role in Crop Productivity

Siderophores are ferric ion-specific chelating agents having the relatively low molecular weight ligand (approximately 20–2000 Da) secreted by bacteria and fungi growing under low iron stress. Its basic function is to solubilize iron from the surrounding environment, which present in the less soluble form to make mineral. They have a variety of roles in pathogenesis, clinical applications, and agriculture. Most of the aerobes and facultative anaerobes are able to synthesize at least one type of siderophore under any stressful condition. For example, all *Penicillium*, *E. coli*, and basidiomycetous fungus *Ustilago maydis* have the ability to produce prototypical hydroxamate-type siderophore. Certain enterobacteria produce prototypical catechol-type siderophore and citrate hydroxamate siderophores (Chu et al. 2010; Hider and Kong 2010).

2.7 Marine Siderophores

Marine bacteria and algae require micromolar concentration of iron. The total iron concentration in the thermocline (the transition layer between layer at the surface and the deepwater layer up to 100 m in the ocean) is only 0.001–2.0 nM. Therefore, many marine bacteria help marine algae (green dinoflagellate *Noctiluca miliaris*) to take iron in mutualism (Amin et al. 2012; Basu et al. 2013). This helps in increased productivity of phytoplankton in the Arabian Sea. These marine bacteria belonging to alpha- and gamma-Proteobacteria produce siderophores, which solubilize iron helping numerous phytoplanktons and other flora in iron uptake. Marine siderophores can be grouped as:

1. Siderophores having different length of a fatty acid appendage that are secreted by marine bacteria as suites of amphiphiles (Ito and Butler 2005; Martin et al. 2006; Homann et al. 2009)
2. Siderophores having the α-hydroxy carboxylic acid moiety, which is photoreactive when combined with ferric (Fe^{3+}) ions (Barbeau 2006; Kupper et al. 2006)

Additionally, many other marine microorganisms produce different siderophores such as *Marinobacter hydrocarbonoclasticus* producing petrobactin; *M. aquaeolei*, petrobactin sulfonates; *Aeromonas hydrophila*, amonabactins; *Bacillus anthracis*, mono- and disulfonated derivatives of petrobactin, and fish pathogens such as *Vibrio anguillarum*, vanchrobactin and anguibactin (Sandy and Butler 2009). Moreover, many species are capable of siderophore production and regulation of metal ions such as *Alcaligenes faecalis* BCCM ID2374 in the succinic acid medium under low iron stress and *Bacillus megaterium* ATCC 19213 producing two types of hydroxamate siderophore under iron-limited conditions, i.e., schizokinen and N-deoxyschizokinen. These siderophores produced under insufficient iron environment are sufficient to absorb iron, aluminum through the siderophore transport receptors and supply to other organisms (Hu and Boyer 1996).

3 Perspectives

Fast-growing human population has increased demand for food. The present approach of agricultural practices and food production methods has high inputs of nitrogen and phosphate, and pesticides are not suitable and sustainable because of high costs and much concern about safety, environmental pollution, and global warming. The use of naturally occurring soil microorganisms and marine microorganisms (van der Heijden et al. 2006a, b) for increasing productivity of crop plants/food, especially in a nutrient-poor ecosystem, is an attractive, eco-friendly, cost-effective, and sustainable alternative to the chemical fertilizers and pesticides. Microbial symbiotic and non-symbiotic cells have multiple beneficial mechanisms. A variety of microorganisms such as biofilms of biofertilizers/PGPR, AM-plant symbiosis, atmospheric N_2-fixing bacteria, and siderophore-producing bacteria and fungi are known. These are responsible for 5–80% of all nitrogen and 75% of phosphorus uptake by plants annually. Such polymicrobial multiple beneficial functions include phosphate solubilization (Hotta et al. 2010; Borriss 2011), biocontrol (Saraf et al. 2014), N_2 fixation (Chauhan et al. 2015), and other plant growth-promoting properties, which is a new positive development in this area (Dodd and Ruiz-Lozano 2012; Mohammadi and Sohrabi 2012; Mitter et al. 2013; Schnurr et al. 2013). The identified and known natural microbes in soil are limited in number. This problem can be overcome by developing inoculum of biofilm and consortia of biofilm for inoculum production on pilot/large scale. Then this can be transferred to the soil to remove operational variables in the cultivation of crop plants for increased crop production.

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Bacterial Quorum Sensing (QS) in Rhizosphere (Paddy Soil): Understanding Soil Signaling and N-Recycling for Increased Crop Production

Bhagwan Rekadwad and Chandrahasya Khobragade

Abstract The multispecies communication in the environment exists as quorum sensing (QS). It is influenced by cell density and the production rate of sensory molecules. Numerous bacteria and other microorganisms have cellular communication through these molecules. It gives an idea about the coevolution in the rhizosphere. The QS supply utilizable form of nitrogen (N), solubilize phosphate, and induce systematic resistance in plants or suppress pathogenic bacteria in the rhizosphere. It is noticed that the conversion of high-molecular-weight N^{-} into low-molecular-weight N^{-} depends on cell density (biofilm) and their behaviors in rhizospheric soil. Thus, QS may be a control point in the rhizosphere (paddy soil) N-mineralization (i.e., N-recycling).

Keywords Paddy • Soil reduction • AHLs • Bacterial community structure • Crop productivity

1 Introduction

Ecologically, in an environment, organisms are related to each other through a relationship. Firstly, when an organism causes harm or kills another organism, it is called antagonistic relationship. Secondly, the organisms in the vicinity are members of an association in which they cooperate and help each other, in such a situation that each receives benefits exceeding their own. This is the so-called synergistic relationship. During symbiosis, the individual organisms live together, have physical contact, and acquire nutrients, becoming interdependent (Farrar et al. 2014; Vassilev et al. 2015).

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Table 1 QS signal molecules produced by bacteria

Organism	QS signal molecules	References
Pseudomonas aeruginosa	C_4-HSL	Veliz-Vallejos et al. (2014)
P. aeruginosa	3-Oxo-C_{12}-HSL	
Sinorhizobium meliloti AK 631	C_6-HSL	
S. meliloti Rm41	C_8-HSL	
	3-Oxo-C_8-HSL	
	C_{16}-HSL	
S. meliloti Rm1021	C_{14}-HSL	
	3-Oxo-C_{14}-HSL	
	C_{16}:1-9 cis-(L)-HSL	
	C_{18}-HSL	
	C_{12}-HSL	
S. meliloti AK 631	C_{10}-HSL	
Agrobacterium vitis	C_{14}:1-9-cis-(L)-HSL	
Agrobacterium vitis	3-Oxo-C_{16}:1-11cis- (L)-HSL, long-chain AHLs	
Aeromonas hydrophila	BHL and HHLg	Defoirdt et al. (2010)
Aeromonas salmonicida	BHL and HHLg	
Burkholderia vietnamiensis	HHLg	
Erwinia amylovora	AI-2	
Fusobacterium nucleatum	AI-2	
Photobacterium phosphoreum	OH-OHLg	
Porphyromonas gingivalis	AI-2	
Prevotella intermedia	AI-2	
Pseudomonas aeruginosa	OdDHLg	
Vibrio campbellii	AI-2, OH-BHLg, CAI-1	
Vibrio harveyi	CAI-1, AI-2, OH-BHLg	
Vibrio salmonicida	AHL	
Vibrio vulnificus	AI-2	
Burkholderia cepacia	C_8HSL	Kalia et al. (2014b)
Burkholderia pseudomallei	C_8HSL, C_{10}HSL, OHC_8HSL, OHC_{10}HSL, $3OC_{14}$HSL, C_8HSL, C_{10}HSL, $3OHC_8$HSL, $3OHC_{10}$HSL	

(continued)

Table 1 (continued)

Organism	QS signal molecules	References
Erwinia carotovora	$3OC_6HSL$	
Bacillus cereus	Peptide PapR	
Staphylococcus aureus	Autoinducing peptide	
Rhizobium leguminosarum	OHC_6HSL, $3OC_6HSL$, C_7HSL, $3OHC_{14}HSL$	
Sinorhizobium meliloti	$C_{14}HSL$ to $C_{18}HSL$	

Microorganisms such as chemo-heterotrophic bacteria, fungi, and mycorrhizae and their metabolic products when adhering to the biotic and abiotic surfaces as a single or multi-cellular assembly can be described as biofilms. This exploitation of communities elaborated a system of intercellular communication, i.e., bacteria respond to the density of nearby bacteria by utilizing signal and receptor molecules (gene expression), which facilitated their adaptation to changing environmental conditions. This process is often termed as quorum sensing (QS). QS system utilizes *N*-acyl-homoserine-lactone (AHL) as the signaling molecules (Table 1) for effective intercellular communication (Huma et al. 2011; Kalia et al. 2011). It is evident that AHLs and autoinducer (AI-I and AI-2) are produced by bulk soil bacteria (Gram positive and Gram negative) and proteobacteria. Many important groups of plant pathogens use QS for regulation of virulence (Crépin et al. 2012; Tortora et al. 2012; Kalia 2013; Kalia et al. 2014b; Kumar et al. 2013; Kalia Pandey et al. 2013).

2 Bacterial Communities Inhabiting Rhizosphere (Paddy Soil)

Paddy is cultivated in upland and lowland areas. Lowland paddy fields have remarkable soil reduction because of flooding. These environmental factors create the anaerobic environment in submerged paddy soil. This created anaerobic condition rhizosphere provides favorable niche for beneficial soil bacteria which aids plants to carry out several important biological activities and ecological processes for their health (Rockstrom et al. 2009; Seefeldt et al. 2009; Godfray et al. 2010). They supply ample organic N, solubilize phosphate, and induce systematic resistance in plants or suppress pathogens inhabiting lowland paddy soil (Emerenini et al. 2015; Tan et al. 2015). This natural process is significant from the agronomic point of view.

The metagenomic studies such as 16S rRNA library, PCR-DGGE, RFLP profiles, and phylogenetics revealed that these submerged paddy soils (rhizospheric soils) are often dominated by both facultative (micro-aero-tolerant) and strict

anaerobic arbuscular mycorrhiza (AM); bacteria including *Clostridium* sp., *Streptococcus* sp., and *Staphylococcus* sp.; and methanogenic archaea (Oldroyd and Downie 2006; Parniske 2008; Banba et al. 2008; Gutjahr et al. 2008; Beatty and Good 2011; Maillet et al. 2011; Sessitsch et al. 2012). These results indicate that paddy soil have the highest diversity of proteobacteria. Obviously, only a few groups of microorganisms belonged to firmicutes followed by acidobacteria and bacteroidetes. This community structure is different from another niche only because of huge application of organic fertilizers which changed nutrient contents and physiological texture of paddy soil. Additionally, the microbial community structure also depends on the change in the seasons. Moreover, effects of organic fertilizer application, soil type, and physiological characteristics of rhizosphere strongly affect the appearance of soil bacterial communities (Kim et al. 2008; Arjun and Harikrishnan 2011; Doi et al. 2011; Peng et al. 2015).

2.1 Diazotrophic Bacteria in Rice Field

Paddy requires N^- as a main limiting nutrient. It utilizes about one-third of the chemical fertilizer from total supply and remaining N^- will be supplied by N-fixing diazotrophic bacteria (Ahemad and Kibret 2014; Geddes et al. 2015). These bacteria include Azospirillum, Burkholderia, Herbaspirillum, and Pseudomonas suppling additional solubilized inorganic phosphate/produce indole acetic acid (IAA) are collectively called as plant growth-promoting rhizobacteria (PGPR). These biofilm-forming communities show QS and are often used as biofertilizers (López-Guerrero et al. 2012; Dwivedi et al. 2015). Moreover, other rhizosphere microorganisms such as *Azospirillum*, *Brevundimonas*, *Bacillus*, *Herbaspirillum*, *Paenibacillus*, *Serratia*, and *Xanthomonas* produce IAA, ethylene, and gibberellin and enhance rice growth through phytostimulation which is the supportive activity exhibited by these bacteria to increase paddy productivity along with N^- fixation, phosphate solubilization, or siderophore production (da Silva Araújo et al. 2013; Liang et al. 2014; Chauhan et al. 2015; Zhang et al. 2015c). The significance of adding these microorganisms includes the increase in seed germination rate, weight, and length of the plant. The use of these microorganisms helps to control 70% of diseases of rice crop and saves 5–30% of lost every year depending on the rice cultivar, pathogen, year, zone, etc. (Oldroyd and Dixon 2014). The main rice pathogens such as *Rhizoctonia solani*, *Magnaporthe oryzae*, and *Xanthomonas oryzae* pv. *oryzae* that are responsible for the blast blight, sheath blight, and bacterial leaf blight on rice plants, respectively, were controlled. The predominant PGPR genera such as *Bacillus* and *Pseudomonas* have anti-pathogenic activity by producing antibiotics, quitinases, siderophores, and proteases against several groups of fungi and bacteria. Thus, the use of these control measures results in reduction of the severity of diseases up to 90% (Han et al. 2005; Rashedul et al. 2009; Filippi et al. 2011; Pérez-Montano et al. 2014; Rekadwad 2015).

3 QS and N-Recycling

In rhizosphere, like root nodules formed in *Glycine max* and *Vigna radiata* (Fig. 1), rice root nodules were formed through interaction in between N-fixing bacteria (rhizobia) and legumes. These rhizobia in symbiotic association synthesize and perceive AHL QS signals (Perrine et al. 2005; Sanchez-Contreras et al. 2007; Oldroyd and Downie 2008; den Camp et al. 2011; Vijayan et al. 2011; Singh et al. 2015). This signaling in biofilm triggers synthesis of exopolysaccharide, which is important for bacterial invasion, increase of nodulation efficiency, mobile DNA (plasmid) transfer, regulation of *nif*-genes, and swarming behavior (Fujishige et al. 2008; Veliz-Vallejos et al. 2014; Mukherji and Prabhune 2015). A carbon sequestration technique (biochar) is applicable for various analyses, viz., carbon storage detection and sequestration, i.e., alteration in decomposition of organic matter rate and plant biomass production (Grover et al. 2015). The process of biochar sorption of *N*-3-oxo-dodecanoyl-L-homoserine-lactone (ODHSL) and AHL indicates that produced intercellular signaling molecules were used by many Gram-negative bacteria for regulation of gene expression (Degrassi et al. 2007; Kalia et al. 2014a; Kumar et al. 2015; Kalia 2015; Kalia and Kumar 2015a, b; Rekadwad 2014; Rekadwad and Khobragade 2015). The biochar disrupts communication within a growing multicellular system (sender cells) busy in AHL synthesis, and the emission of green fluorescent protein (GFP) produced in response to an AHL signal by receiver cells has been stopped (Natrah et al. 2011). The rate of disruption in cell-cell communication will be changed, with biochar prepared at 1292 °F (surface area (*A*) of 301 m^2/g). This results in the inhibition of cellular communication up to tenfold, which is more than an equivalent mass of biochar prepared at 572 °F (*A* of 3 m^2/g). Moreover, it is noticed that the gene expression affected by biochar depends on intercellular signaling. This method also regulates the various microbial processes in soil such as N^- fixation and reduces attack of pest on crops (Masiello et al. 2013; Devi and Saroha 2015; Bogusz et al. 2015; Wang et al. 2015a, b; Zhanf et al. 2015; García-Jaramillo et al. 2015; Gul et al. 2015).

An ecosystem possesses a huge quantity of macromolecular organic N in the form of chitin followed by different structural and functional proteins, LINGO proteins, and nucleic acids (NA). This means plants have limited access to the organic N^-, utilize only organic monomers having low molecular weight (MW), and primarily rely on products of N-mineralization (inorganic N-compound) such as ammonium (NH_4^+) and nitrate-N. In nature, polymeric organic N^- is converted to monomeric organic N^- through N-mineralization, i.e., acquisition and N-uptake by microorganism and plant biomass and their biomineralization (through enzymatic conversion) into monomers as predation, carbon starvation (Merchant and Helmann 2012; Schnurr et al. 2013), biochar, and water-potential fluctuation. These molecules in paddy soil are actively interchanged between plants and soil bacteria that imply photosynthate fuels generated by plants enhance microbial growth and increase microbial activity under carbon-limited condition. This results in increased rhizosphere N-mineralization. The cell-bound macromolecular organic N^- in the

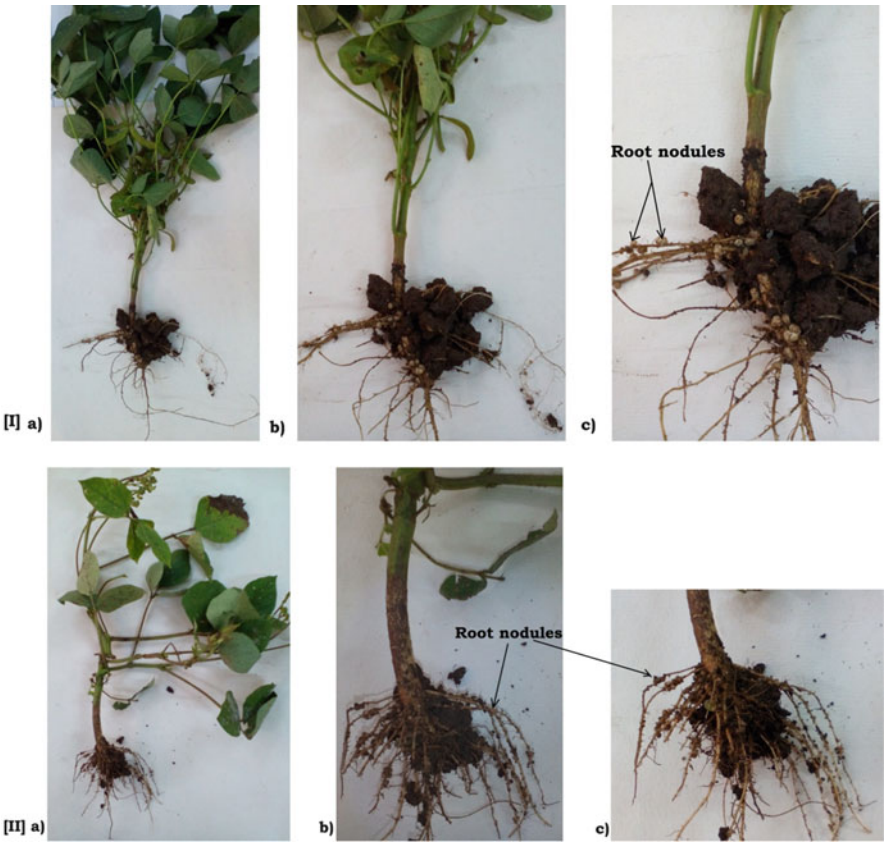

Fig. 1 Root nodules of leguminous plants: [I] *Glycine max* (Soya bean), [II] *Vigna radiata* (Mung)

form of chitin; structural and functional proteins; nucleotides, i.e., NA; and their enzymatic de-polymerization are very slow which limit its availability to plants. It is recorded that QS in rhizosphere is high where rhizospheric chitinase and protease-specific activities are observed (Koutsoudis et al. 2006; Bierke et al. 2008; Xiao and Wu 2014; Balagurumurthy et al. 2015; Zhang et al. 2015a). This results in the remarkable increase of dissolved organic nitrogen (DON) in paddy soil than in bulk soil. The DON is the low-molecular-weight (<3000 Da) organic N-compound, which are undetectable in bulk soil. The rhizosphere comprises 15% of DON. The increase in percentage of DON is directly proportional to the extracellular enzyme production by bacteria and extent of QS or behavior of biofilm in rhizosphere (Gonzalez and Keshavan 2006; Guo et al. 2011; Liu et al. 2013; Hanke et al. 2014; Zhao et al. 2014; Chiang et al. 2015; He et al. 2015; Liu et al. 2015; Song et al. 2015; Wang et al. 2015a, b; Sodano et al. 2016). The increased percentage of DON indicates the increased proteobacterial AHL QS signals in the rhizosphere. It is noticed that the conversion of high-molecular-weight N^{-} into

low-molecular-weight N^- by N-cycling enzymes depends on cell density (biofilm) and their behaviors in rhizospheric soil. Thus, QS may be a control point in the rhizosphere (paddy soil) N-mineralization (i.e., N-recycling) (Madsen et al. 2003; Radutoiu et al. 2003; Feng et al. 2004; Arrighi et al. 2006; Smit et al. 2007; DeAngelis et al. 2008; Tahng et al. 2011; Broghammer et al. 2012; Wang et al. 2014; Zhang et al. 2015b; Zhu et al. 2015).

4 Future Perspectives

The QS is the close coevolution and multispecies communication that exists in the environment. It is strongly regulated by the cell density of microbes in many environments. Sometimes QS is autoregulated by molecules and this mechanism is perceived to the next generation. How it is autoregulated is in its infancy? The QS research needs new discoveries, which will change our views about the multispecies communication, which exists between plants and microorganisms.

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Use of Plant Growth-Promoting Rhizobacteria as Biocontrol Agents: Induced Systemic Resistance Against Biotic Stress in Plants

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Abstract Plant growth-promoting rhizobacteria (PGPR) are free-living bacteria able to colonize roots and soil around them that have a positive effect on plant growth, development, and health. One of the mechanisms by which PGPR exert a beneficial effect involves the capacity to control growth of deleterious organisms diminishing or preventing their negative effects on plant health and growth. Pathogen biocontrol implicates diverse features of bacteria; one of them is the antagonism that excludes pathogen due to the ability of some bacteria to colonize faster and more effectively a niche, reducing nutrient availability for the deleterious organism. Also some bacteria produce antibiotics, organic compounds that are lethal in low concentration for growth and metabolic activities of other microorganisms. Finally, the ability of bacteria to elicit a defense response in plant, called induced systemic resistance (IRS), involves the induction of synthesis of defense metabolites, but without causing a disease itself, enhancing the plant's defensive capacity. This chapter analyzed and discussed PGPR as biocontrol agent and the possibility to use them as ecological alternative to the use of agrochemicals, since they have been proved in different plant species in order to diminish the damage of pathogen and to reduce losses in crops.

Keywords PGPR • Biocontrol mechanisms • Induced systemic response • Siderophores • Antibiotics

1 Introduction

In nature, plants interact with a wide variety of microorganisms including soil bacteria. In the rhizosphere, that is, the soil/root interface, microorganisms are far more abundant than in bulk soil. This is because roots release significant part of

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their photo-assimilates as different metabolites, which are the main source of nutrients for bacteria that stimulate their copiousness in the rhizosphere (Hartmann et al. 2008; Diallo et al. 2011). In return, a number of them are able to exert a beneficial effect on plant growth playing a fundamental role in the adaptation of the plant to the environment (Hallman et al. 1997; Rodríguez and Fraga 1999; Hardoim et al. 2008). Kloepper and Schroth (1978) coined the term plant growth-promoting rhizobacteria (PGPR) to define free-living bacteria able to colonize roots and the soil around them that have a positive effect on plant growth and development (Fulchieri et al. 1993; Compant et al. 2005). Generally, different PGPR are associated with most, if not all, plant species and are present in almost all environments. Therefore, since PGPR were characterized as helpful for plants, different genera like *Azospirillum*, *Herbaspirillum*, *Bacillus*, *Pseudomonas*, *Burkholderia*, and *Gluconacetobacter* have been used to improve seedling establishment, weight enhancement, and yield increase and to help plants to cope with biotic stresses (herbivore and pathogen attack) in different plant species of economic importance (Lugtenberg and Kamilova 2009).

PGPR have direct and indirect mechanisms by which they interact positively with plants; the direct mechanisms are related to plant nutrition and development and include production of plant growth regulators such as abscisic acid (ABA), gibberellins (GAs), and indole acetic acid (IAA) (Bottini et al. 1989; Podile and Kishore 2006; Piccoli et al. 2011; Piccoli and Bottini 2013); nitrogen fixation that contributes to the accumulation of this element in soil; phosphate solubilization that makes P available for plant uptake (Rodríguez and Fraga 1999; Rodriguez et al. 2006); and siderophore production that improves Fe acquisition (Masalha et al. 2000; Miethke and Marahiel 2007). On the other hand, indirect mechanisms involve the capacity of several bacteria to control growth of deleterious organisms and so diminishing or preventing their negative effects on plant health (Haas and Défago 2005). The last mechanism has been defined as biocontrol and includes diverse metabolic features of the PGPR, which are subject of the present chapter.

Diseases caused by pathogens become relevant by affecting economically important species since they are responsible for significant loses in yield. Factually, different agrochemicals as well as genetic tools have been used in order to control diseases, but they are not always effective. Moreover, many agrochemicals are nondegradable and therefore harmful for the environment (Lugtenberg and Kamilova 2009). In the last decades, the use of PGPR as biocontrol agents became an environmentally friendly alternative to deal with diseases, thus decreasing the use of chemicals in agriculture (Gerhardson 2002). Pathogen control involves different aspect and features of bacteria; one mechanism is the pathogen exclusion based on the capability of some bacteria to colonize faster and more effectively a niche, thus reducing the nutrient availability for the deleterious organism. An important aspect of this mechanism includes Fe chelation by bacterial siderophores that makes the element unavailable for the pathogen (Whipps 2001; Podile and Kishore 2006; Singh et al. 2011). Other mechanism is the capacity to produce antibiotics which include a chemically heterogeneous group of organic compounds that are lethal in low concentration for growth and metabolic activities of other

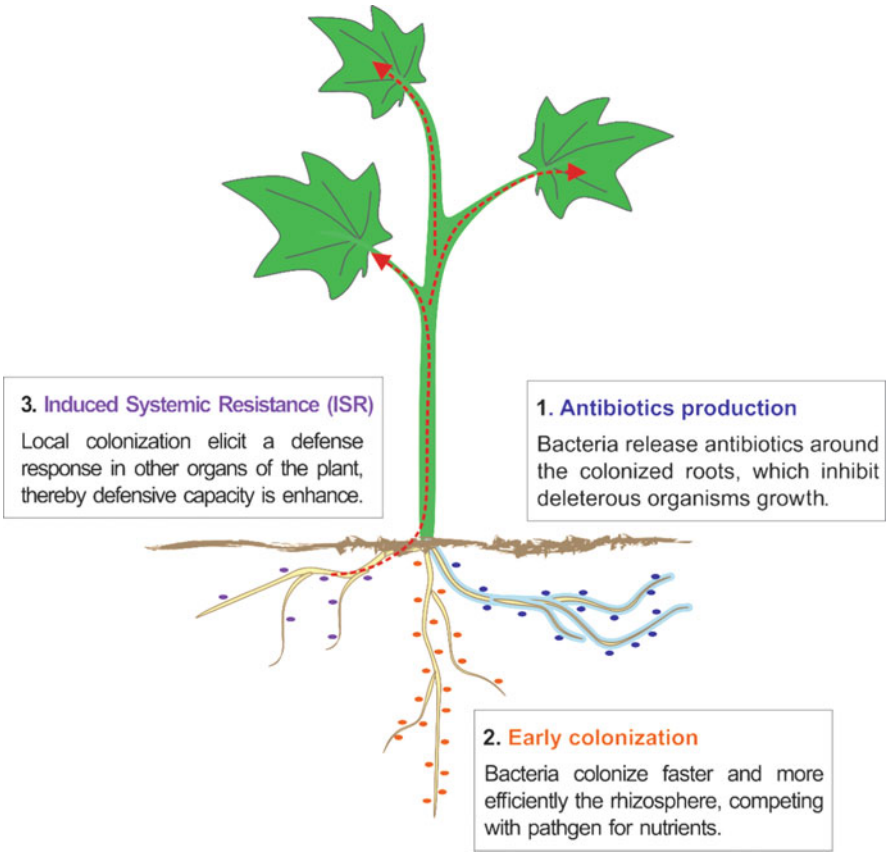

Fig. 1 Mechanisms of disease biocontrol by plant growth-promoting rhizobacteria. Adapted from Lugtenberg and Kamilova (2009)

microorganisms (Haas and Défago 2005). Besides the ability of bacteria to produce diverse compounds, PGPR can reduce the incidence of the pathogen attack by eliciting a defense response in plant, called induced systemic resistance (ISR) (Glick 2015). In these responses, PGPR elicit synthesis of different defense metabolites, but without causing a disease itself, which modify the physical and biochemical properties of the host enhancing the plants defensive capability (Kloepper et al. 2004). Figure 1 shows a diagram of the different mechanisms.

This chapter is focused in reviewing PGPR as biocontrol agents. The following sections are dedicated to different biocontrol mechanisms mentioned above and to analyze the effectiveness of PGPR in biocontrol, as well as the possibility of using PGPR as an ecological alternative in the management of plant diseases. Moreover, the advantages and disadvantages of their use will be discussed in the context of environmental impact.

2 PGPR as Biocontrol Agents

2.1 Antibiotic Production

In agriculture, plant cultivars have been selected in order to optimize crop yield (quantity and quality); therefore, genetic variability and resistance to diseases are frequently decreased in comparison with wild-type ancestors. Plant diseases cause severe losses in crop production, and a variety of chemical pesticides are used to control maladies, but chemicals are often harmful for the environment and farmer's and consumer's health and even for the crops themselves. The excessive use of pesticides also increases resistance of the pathogen with the outcome of resistant strains (Burketova et al. 2015). Taking this in consideration, compounds of natural origin are expected to have lower environmental impact than synthetic pesticides, mainly because they are easier to biodegrade (Couillerot et al. 2014). In such a venue, a more sustainable alternative seems to be the use of microbial pesticides, like metabolites usually produced by bacteria and fungi. Some advantages of microbial antibiotics in comparison to chemical products have been reported. They may have low persistence in the environment, higher specificity against the pathogen target, minor induction of pathogen resistance, and low production cost (for instance, direct infection of the plant with the microbial producer may work). However, scientific evidence indicates that these advantages are not always achieved. On the other hand, disadvantages include that the level of protection can vary between crop species, they are highly influenced by environmental factors, and aspects of biosafety and impact on ecosystems have to be evaluated (Burketova et al. 2015; Bonaterra et al. 2012; Lagerlöf et al. 2015).

Microbial antibiotics are bioactive metabolites produced by bacteria and fungi that in certain concentration suppress disease agents (Fig. 1, item 1). It has been demonstrated, however, that in subinhibitory concentration these products can produce other side effects on the pathogen, such as changes in gene transcription, virulence, motility, and biofilm formation (Raaijmakers and Mazzola 2012). Chemical classes of secondary metabolites reported as having antibiotic effect are diverse, and they are not produced by a single strain since each strain usually produces more than one antibiotic. As is shown in Table 1, *Pseudomonas fluorescens* strains have an important role as biocontrol agents in plants due to their ample production of antimicrobial metabolites. In this section some antibiotics will be considered, especially those reported for *Pseudomonas* spp. and *Bacillus* spp. like 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrins, pyoluteorins, phenazine, iturins (cyclic lipopeptide), and hydrogen cyanide (HCN) (Ahmadzadeh and Tehrani 2009; Blumer and Haas 2000; Costa et al. 2009; Kennedy et al. 2015; Morohoshi et al. 2013; Pieterse et al. 2014).

Phenazines are nitrogen-containing heterocyclic pigmented compounds, known for their broad-spectrum antifungal activity, synthesized by a wide range of bacterial genus, including *Pseudomonas*, *Burkholderia*, *Brevibacterium*, *Streptomyces*, *Nocardia*, *Erwinia*, *Vibrio*, *Pelagiobacter*, and some Actinomycetales like

Table 1 Bacterial antibiotics and pathogen antagonism

Biocontrol agent	Antibiotic produced	Pathogen	Reference
Chaetomium globosum NM0066	Gliotoxin	*F. oxysporum f.* sp. *vasinfectum* *F. graminearum* *F.sulphureum* *Cercospora sorghi* *B. cinerea* *Alternaria alternata*	Li et al. (2011)
Chryseobacterium wanjuense KJ9C8	Hydrogen cyanide	*Phytophthora capsici*	Kim et al. (2012)
Burkholderia cepacia	Pyrrolnitrin	*Rhizoctonia solani*	Hwang et al. (2002)
B. pyrrocinia 2327T	Pyrrolnitrin	*Trichophyton* *Rhizoctonia solani*	Kwak and Shin (2015) Costa et al. (2009)
P. brassicacearum J12	DAPG Hydrogen cyanide	*Ralstonia solanacearum*	Zhou et al. (2012)
P. chlororaphis subsp. *aurantiaca* StFRB508	Phenazine	*F. oxysporum* f. sp. *conglutinans*	Morohoshi et al. (2013)
Pantoea agglomerans	Pantocins Herbicolins Microcins Phenazines	*Erwinia amylovora*	Braun-kiewnick et al. (2012), Smith et al. (2013)
P. fluorescens *P. fluorescens 2-79* *P. fluorescens* Psd *P. fluorescens Pf-5* *P. fluorescens* WCS417r	Phenazine Pyrrolnitrin Pyoluteorin dimethylhexadecylamine DAPG	*Gaeumannomyces graminis var. tritici* *Pythium ultimum* *B. cinerea* *P. syringae* pv. *tomato*	Mazzola et al. (1992) Upadhyay and Srivastava (2011) Howell and Stipanovic (1980) Hernández-león et al. (2015) Weller et al. (2012)
P. aureofaciens 30-84	Phenazine	*F. oxysporum*	Mazzola, et al. (1992)
P. protegens	DAPG		Ramette et al. (2011)
Bacillus subtilis	Iturin Fengycin	*Podosphaera fusca*	Romero et al. (2007)
B. amyloliquefaciens	Iturin A	*Rhizoctonia solani*	Yu et al. (2002)
B. thuringiensis UM96	Chitinase	*B. cinerea*	Martínez-absalón et al. (2014)

Streptomyces (Kennedy et al. 2015; Mavrodi et al. 2010; Morohoshi et al. 2013). The principal effect of phenazines is to generate reactive oxygen species (ROS) and to uncouple oxidative phosphorylation. Although these effects increase virulence and pathogenesis, the primary role of phenazines is as antibiotic that inhibits fungal pathogens; they also induce protein of defense pathways, iron chelation, biofilm formation, and modulation of gene expression (Pierson and Pierson 2010). Other antibiotics are pyrrolnitrins and pyoluteorins, which are tryptophan-derived metabolites produced mainly by Gram-negative bacteria, as *Pseudomonas* spp.

Burkholderia spp., and *Serratia* spp. Pyrrolnitrins are monochlorinated heteroaromatic pyrrole rings, while pyoluteorins possess dichlorinated rings (Pang et al. 2015; Schmidt et al. 2009). No mode of action has been published for pyoluteorin, although pyrrolnitrin has been reported to inhibit respiratory electron transport and synthesis of proteins; they also combine with cell membrane phospholipids, thus affecting transport (Haas and Défago 2005; Nose and Arima 1969; Tripathi and Gottlieb 1969). DAPG is a benzenetriol in which two of the ring hydrogens are replaced by acetyl groups. Troppens et al. (2013) demonstrated that DAPG produced by *Pseudomonas fluorescens* alters mitochondrial morphology, with loss of the membrane potential and increase of cytosolic Ca_2^+ in *Neurospora crassa*. Hydrogen cyanide is generated due to oxidation of glycine that produces HCN and CO_2 by a membrane-bound flavoenzyme (HCN synthase). It is a potent inhibitor of cytochrome c oxidase and several other metalloenzymes (Blumer and Haas 2000). The HCN and pyrrolnitrins produced by *Pseudomonas chlororaphis* strain PA23 have repellent and nematicidal activity against *Caenorhabditis elegans* (Nandi et al. 2015). Iturins are lipo-polypeptides (heptapeptides) with a β-amino fatty acid that exhibit strong antifungal activity (Arrebola et al. 2010). These antibiotics have been studied mainly in *Bacillus* spp., and it has been proposed that the antimicrobial activity of iturins relies predominantly on their capability to augment membrane permeability due to strong interaction with the phospholipids and sterols (Zhang et al. 2013). Figure 2 shows the structure of some of the mentioned antibiotics.

Generally, pesticides produced by microbes may play an important role in sustainable agriculture, with competitive advantages, although their application at the moment is limited. These antibiotics are lesser aggressive to environment than synthetics, and also application of biocontrol microorganism has some other advantages that benefit crops like nutrition increase and growth promotion. However, the knowledge of the mechanisms for microbial biocontrol is a key factor to achieve. There is limited evidence so far regarding in situ antibiotics produced by soil bacteria at concentrations to achieve anti-pathogenic effects.

2.2 Nutrient Competition and Siderophore Production

Another mechanism of phytopathogen antagonists is production of siderophores (Beneduzi et al. 2012). Under limited conditions bacteria with high capability to compete for nutrient uptake can restrict growth of minor contenders. In the rhizosphere, competition for niches, also called niche exclusion, has been described as a mode of biocontrol (Beattie 2007). In roots surface exist niches with high nutrient content (as it was mentioned root exudates are rich in amino acids, monosaccharides, and organic acids), which are attractive to organisms, including pathogens. PGPR compete with other microorganisms including pathogens for these nutrients, although not killing them (Fig. 1, item 2). For example, *Kloeckera apiculata* strain 34–9, isolated for its biocontrol effectiveness against *Penicillium italicum*,

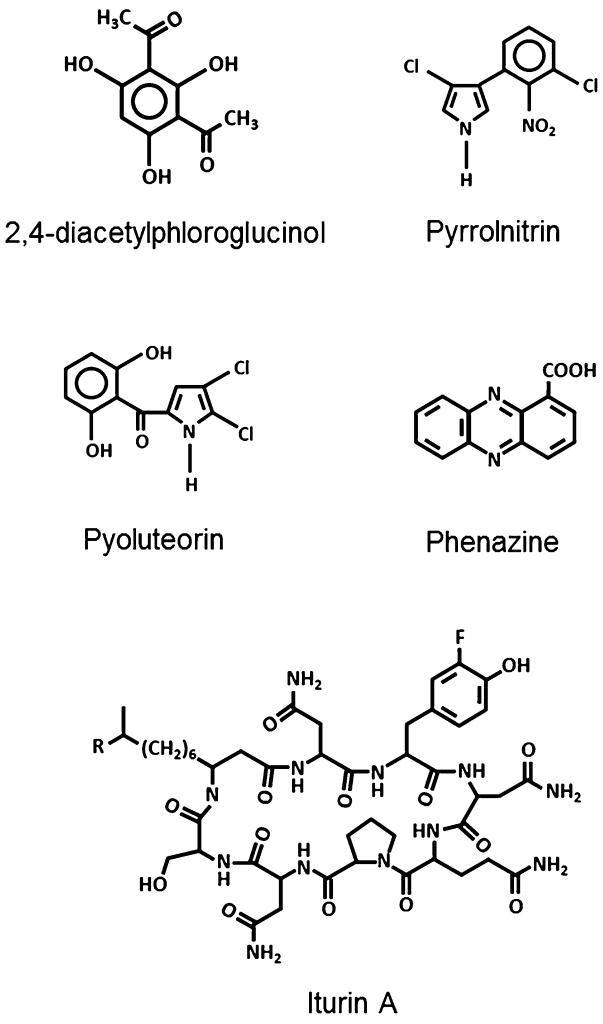

Fig. 2 Examples of antibiotic structures produced by PGPR. Each structure was redrawn from Moran et al. (2009), Haas and Défago (2005), and Pang et al. (2015)

competes with the phytopathogen for nutrients and vitamins, inhibiting conidial germination of the pathogen albeit it is not able to kill the spores (Liu et al. 2013). Bencheqroun et al. (2007) provided in vitro and in situ evidence for nutrient competition in apple (mainly amino acids), as the most important mechanism of biocontrol activity of *Aureobasidium pullulans* strain Ach1-1 against *Penicillium expansum* on harvested apple fruits. Also, competition for nitrogen and carbon sources, secretion of hydrolytic enzymes, and elicitation of ISR play an important role in the biocontrol mechanism of *Pichia guilliermondii* M8 against *Botrytis cinerea* (Zhang et al. 2011). Regarding nutrient competition, root exudates are not the only factor, since essential soil nutrients like Fe are important too.

Fe is the second most abundant metal in the Earth crust and is essential to almost all organisms, since it has a crucial role in primary metabolism, oxygen transport and respiration, electron transfer, DNA synthesis, oxidative stress responses, and secondary metabolism (Abd-alla 1998; Ams et al. 2002; Braun 2001). Many environments tend to be a limitation and its deficiency is very common. The low Fe bioavailability is due mainly to the low solubility of Fe oxides, especially in alkaline and aerobic conditions. To increase Fe assimilation, organisms developed several strategies, and the most common is the production of extracellular siderophores (Rajkumar et al. 2010; Sahu and Sindhu 2011). Siderophores are low molecular mass compounds with high iron affinity and are typically produced in secondary metabolism by bacteria, fungi, and monocotyledonous plants in response to Fe stress (Gasser et al. 2015). Siderophores can chelate Fe^{+3} with high affinity, solubilizing and extracting it from most mineral or organic complexes. Siderophores have higher affinity to Fe^{+3} than to Fe^{+2}, because it is difficult to discriminate Fe^{+2} from other cations (Cu^{+2}, Zn^{+2}, Mn^{+2}, Ni^{+2}), but there are few biologically important trivalent cations (e.g., Co^{+3}), so in biological media, the selectivity to Fe^{+3} will be more effective (Hider and Kong 2010). There are basically four chemical classes of bacterial siderophores: phenol-catecholates, hydroxamates, rhizobactins, and hydroxycarboxylates. Phenol-catecholates have the highest Fe affinity, but is labile and unstable. Examples of this class are enterobactin, produced by *Escherichia* spp., *Enterobacter* spp., *Pseudomonas* spp., *Bacillus* spp., and *Agrobacterium* spp.; mycobactin, produced by *Mycobacterium* spp., *Nocardia* spp., and *Rhodococcus* spp.; and pyochelins, produced by *Pseudomonas* spp. On the other hand, hydroxamates are divided in citrate type (schizokinen, aerobactin), produced by *Bacillus* spp., *Aerobacter* spp., *Enterobacteriaceae* spp., and *Arthrobacter* spp., and ferrioxamines, produced by *Actinomyces* spp., *Streptomyces* spp., *Nocardia* spp., and *Arthrobacter* spp. Rhizobactins are produced by nodular bacteria, and finally hydroxycarboxylates, like pyoverdine (Pvd), are produced principally by *Azotobacter* spp. and *Pseudomonas* spp. Fungal siderophores mostly include three types: the rhodotorulic (hydroxamates), ferrichrome type, and fusarinines. Almost all fungal siderophores are hydroxamates, e.g., *Penicillium* spp. and *Aspergillus* spp. (Crowley 2006; Liu et al. 2015; Zheng and Nolan 2012; Raines et al. 2015). See Fig. 3 for siderophores structures.

As PGPR can improve plant health by acting as antagonists of pathogens by mechanisms such as solubilizing Fe and P, N fixation, antibiotic, and hormone productions, some evidences show that competition for Fe, N, and carbohydrates on leaves could limit the conditions for infection processes (Halfeld-vieira et al. 2015; Parangan-Smith and Lindow 2013; Smith et al. 2013). Siderophore production by PGPR confers them competitive advantages to colonize roots and exclude other microorganisms from this ecological niche. Some siderophores, like pyoverdine (Pvd), might compete at a distance depriving pathogens of Fe, thus showing bacteriostatic and fungistatic activities (Haas and Défago 2005). The ability to acquire Fe by microbial siderophores may determine the ability of the bacteria to

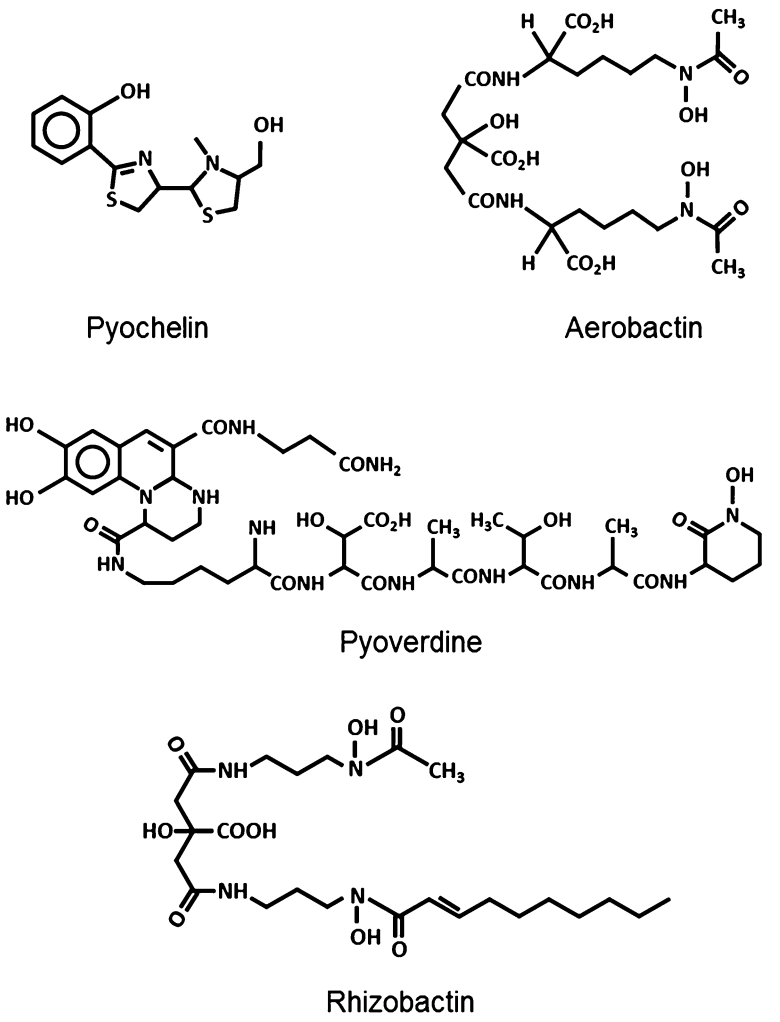

Fig. 3 Examples of siderophore structures produced by PGPR. Each structure was redrawn from Zheng and Nolan (2012), Hider and Kong (2010), and Permark et al. (1993)

compete for nutrient, like C source and even Cu or Zn, enhancing iron uptake by plant too (Beneduzi et al. 2012).

The genus *Pseudomonas* is one of the most studied as control disease agent because its ability to produce siderophores. Kloepper et al. (1980) suggested that disease suppression is caused in part by microbial pseudobactin produced by *Pseudomonas fluorescens* strains. Siderophores efficiently complex Fe (III) in soils, making it unavailable to pathogens, inhibiting their growth. *Arthrobacter* spp., *Curtobacterium* spp., *Enterobacter* spp., *Microbacterium* spp., *Pseudomonas* spp., or *Stenotrophomonas* spp. presented antagonism against the pathogen

Xanthomonas axonopodis pv. passiflorae, which is explained by competition for Fe and N-compounds on leaves of passion fruit (Halfeld-vieira et al. 2015). The catecholic siderophore producer *Bacillus subtilis* CAS15 significantly inhibited the mycelial growth of 15 different plant pathogens of *Fusarium* spp., *Colletotrichum* spp., *Pythium* spp., *Magnaporthe* spp., and *Phytophthora* spp. and also showed plant growth promotion effects in pepper. After treatment with Fe, the suppression by CAS15 on *Fusarium* wilt was significantly diminished, which indicate siderophore production as control mechanism (Yu et al. 2011). Finally, the effects of siderophores on control of phytopathogens may not be direct; De Vleesschauwer et al. (2008) showed the ability of *Pseudomonas fluorescens* WCS374r to trigger induced systemic responses in rice. They found that pseudobactin-type siderophore was responsible for ISR elicitation. Application of WCS374r-derived pseudobactin in roots activated multiple defense responses, enhancing resistance level against the leaf blast pathogen *Magnaporthe oryzae* (Table 2).

In summary, several bacteria develop iron chelating mechanisms that capture Fe from the environment and make it unavailable to competitors. Several studies indicate that control of plant diseases by using siderophore-producing bacteria could be a better remedy than administering antibiotics. Siderophores have also been examined for their role in plant Fe acquisition and for their capacity to mobilize heavy metals. Rhizospheric microorganisms are ideal as biocontrol agents, since they could reduce environmental impact and application costs, replacing chemical compounds as well as promoting plant nutrition.

Table 2 Bacterial siderophore and pathogen antagonism

Biocontrol agent	Siderophore produced	Pathogen	Reference
Arthrobacter *Curtobacterium* *Enterobacter* *Microbacterium* *Pseudomonas* *Stenotrophomonas*		*Xanthomonas axonopodis* pv. *passiflorae*	Halfeld-vieira et al. (2015)
Rhodotorula glutinis	Rhodotorulic acid (hydroxamate)	*Penicillium expansum*	Calvente et al. (1999)
P. putida Pp17		*Ralstonia solanacearum*	Kheirandish and Harighi (2015)
P. putida	Pseudobactin	*F. oxysporum* f. sp. *lini* *Gaeumannomyces graminis* var. *tritici*	Kloepper et al. (1980)
P. fluorescens WCS374r	Pseudobactin	*Magnaporthe oryzae*	De Vleesschauwer et al. (2008)
B. subtilis	Catecholate	*Fusarium*	Yu et al. (2011)

2.3 Induced Systemic Resistance (IRS)

Plants respond to local herbivore or pathogen attack by synthesizing de novo compounds in order to inhibit or at least reduce its incidence (Heil and Bostock 2002). There are some bacteria able to elicit similar responses that increase the defense and resistance of the plant against viral, bacterial, and/or fungal pathogens, but without causing a disease by themselves. As mentioned before, this is called induced systemic resistance (IRS) and is one of the mechanisms included in biocontrol (Fig. 1, item 3). When PGPR have elicited an IRS, the basal defense of the plant is enhanced; so after a pathogen infection this is evidenced with reduced rate of disease development, and in consequence, a lesser number of plants are affected, and/or the severity of the damage is lower (Van Loon et al. 1998; Kloepper et al. 2004). Thereby, PGPR are capable to pre-sensitize plant cell metabolism, and by consequence these plants are able to respond faster and more effectively when they are expose to a biotic stress than those that were not previously stimulated (Compant et al. 2005; Harish et al. 2008). This phenomenon is called "priming," and although it is a different protective mechanism against pathogen attack as compared with direct defense, both have a similar phenotypic response (Verhagen et al. 2011).

Bacteria release different compounds in the rhizosphere that act as elicitors, and some of them are perceived by the plant roots as signals that trigger defense responses (Gray and Smith 2005; Bais et al. 2006); to make it happens, plant roots have to be capable to perceive and recognize those elicitors similarly to how they perceive pathogen elicitors (Van Loon 2007; Thakur and Sohal 2013). These compounds belong to different chemical groups such as polysaccharides, lipids, glycopeptides, glycoproteins, and volatiles, and even antibiotics and siderophores can act as elicitors (Van Loon and Bakker 2005; Thakur and Sohal 2013). After they are perceived by plant roots, signal transduction pathways are activated to trigger the synthesis of different phytoalexins, defense enzymes, pathogenesis-related proteins (PR proteins), and cell wall reinforcement (Liu et al. 1995; Van Loon 1997; Heil and Bostock 2002; Magnin-Robert et al. 2007). An important feature of the ISR is that the bacteria responsible of eliciting the response must stay spatially separate of the pathogen, for example, the bacteria interact with roots while the response is located in the aerial part of the plant where the pathogen is located (Liu et al. 1995; Van Loon 2007; Rudrappa et al. 2010).

ISR has been studied in several species, both model plants (like *Arabidopsis thaliana*), and economic important crops summarized in Table 3. In those studies, *Pseudomonas* spp. and *Bacillus* spp. have been mainly used (Liu et al. 1995; Haas and Défago 2005; Kloepper et al. 2004; Van Loon and Bakker 2005; Van Loon 2007; Liu et al. 2009; Podile and Kishore 2006). As examples, in *A. thaliana*, *Bacillus* spp. triggered IRS by releasing 2,3-butanediol, while *Pseudomonas* spp. by production of 2,4-diacetylphloroglucinol (Iavicoli et al. 2003; Ryu et al. 2004). In grapevine, Salomon et al. (2014) have shown that root inoculation with *P. fluorescens* as well as *B. licheniformis*, besides improving plant growth, elicited

Table 3 Induced systemic resistance elicited by plant growth-promoting rhizobacteria

PGPR strain	Plant species	Disease (pathogen)	Reference
B. subtilis GB03 *B. subtilis* Sb4-23, Mc5-Re2, Mc2-Re2 *B. amyloliquefaciens IN937a* *B. mycoides* *B. cereus AR156* *B. lipopeptides*	*Arabidopsis* Sugar beet Tomato	*Erwinia carotovora* subsp. *carotovora* *Cercospora beticola* *P. syringae* pv. *tomato* *Polymyxa betae* *Meloidogyne incognita*	Ryu et al. (2004) Bargabus et al. (2002) Niu et al. (2011) Desoignies et al. (2013) Adam et al. (2014)
P. fluorescens CHA0 *P. fluorescens*	*Arabidopsis* Tomato	*P. syringae* pv. *tomato* *Peronospora parasitica* *Meloidogyne javanica*	Pieterse et al. (1998) Iavicoli et al. (2003) Siddiqui and Shaukat (2004) Weller et al. (2012)
P. fluorescens *B. licheniformis*	Grapevine	*B. cinerea*	Salomon et al. (2014)
P. fluorescens 89B61 *P. aeruginosa* 7NSK2	Tomato	*Phytophthora infestans* *B. cinerea*	Yan et al. (2002) Audenaert et al. (2002)
P. putida BTP1	Bean	*B. cinerea*	Ongena et al. (2004)
Pseudomonas *Pantoea* *Acinetobacter*	Grapevine	*B. cinerea*	Verhagen et al. (2010) Magnin-Robert et al. (2013)

de novo synthesis of the monoterpenes α-pinene, terpinolene, 4-carene, limonene, eucalyptol, and lilac aldehyde A, as well as the sesquiterpenes α-bergamotene, α-farnesene, nerolidol, and farnesol in leaves. The synthesis of terpenes in plant tissues have been associated with defense responses to deleterious organisms (Neri et al. 2006; Leitner et al. 2008; Escoriaza et al. 2013), and their antimicrobial properties have been demonstrated (Machida et al. 1998; Brehm-Stecher and Johnson 2003; Semighini et al. 2006; Park et al. 2009). Also, other studies showed that grapevine leave extracts from inoculated plants have enhanced antioxidant properties due in part to terpenes (Salomon et al. 2016). Moreover, strains of *Pseudomonas* spp., *Pantoea* spp., and *Acinetobacter* spp. elicited synthesis of chitinases and β-1,3-glucanases that enhance tolerance to *B. cinerea* infection in leaves, reducing the lesion diameter (Verhagen et al. 2010; Magnin-Robert et al. 2013). In other crops as bean, a nonpathogenic *P. putida* strain increased hexenal levels (volatile antifungal compound) and the expression of enzymes involved in its synthesis, thereby reducing the incidence of *B. cinerea* infection (Ongena et al. 2004). In tomato, different strains of *Pseudomonas* spp. elicited IRS against both fungal and nematode diseases (Audenaert et al. 2002; Yan et al. 2002; Siddiqui and Shaukat 2004). Additional examples are shown in Table 3.

3 Conclusion

In literature, there is a plethora of information about PGPR as biocontrol agents in which bacteria have shown positive effects in a variety of crops. All the mechanisms have been extensively analyzed, mainly in short-scale trials. The use of natural products as antibiotics and/or siderophores as well as the capability to elicit ISR by bacteria is a positive ecological alternative to the use of agrochemicals, since there are evidences that the impact on environment is lower as compared with agrochemicals. But besides the extent literature about this topic, other studies about impact on the environment and the potential large-scale use of these technologies are still needed. In order to be able to commercialize PGPR to control diseases, biosafety and environmental impact have to be deeply analyzed, and regulation rules established accordingly. In this regard, several features of the biocontrol agents released to the environment, such as stability, impact on other beneficial microorganisms, and effect on human health, have to be considered for adequate implementation and use.

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Biological Routes for the Synthesis of Platform Chemicals from Biomass Feedstocks

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Abstract Development of processes to produce fuels and chemical from biomass offers an exciting opportunity to achieve a sustainable supply from renewable sources as compared to fossil fuel-based methods. Technologies relying only on chemical catalytic routes have shown limitations in achieving the desired yield of a product molecule. Therefore, recent developments in research have emphasized the importance of integrating a biocatalytic route to a chemo-catalytic route to produce commodity chemicals with high conversion and selectivity. Microorganisms including bacteria, fungus and algae are versatile in nature and have potential to yield platform molecules which can be upgraded to produce their petrochemical counterparts. Genetic engineering techniques combined with metabolic flux analysis are employed to further enhance the productivity. On subsequent purification, the platform molecule may be used as a reactant for chemo-catalytic processing to produce a range of high-value chemicals. In this way, a novel integrated fermentation and catalytic processing strategy is envisaged, which will open avenues for producing chemicals from renewable sources. The chapter covers the progress made in this direction by summarizing the routes for producing a platform molecule via biocatalytic transformations.

Keywords Biomass • Fermentation • Catalysis • Platform chemicals

1 Introduction

The development of technologies for establishing a successful biorefinery are most sought after to substitute the existing petrochemical technologies. At present, about 92% of commodities are produced from petrochemical sources (Reed 2012), such

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as benzene, toluene, xylene, 1,3-butadiene, propylene, ethane and methane (Boisen et al. 2009), etc., to provide for applications in food, textiles, household products, transportation and other goods. The depletion in fossil fuel reserves has therefore necessitated the search for renewable sources to sustainably produce fuels and chemicals. Therefore, the market for renewable chemicals is estimated to increase from $57.5 billion (in 2012) to $83.4 billion by 2018 and is expected to grow from 2% to 22% by 2025 (Bergeson and Roberts 2013). The drivers for such growth are concerted efforts to reduce dependency on crude oil, capacity building for a less volatile and reliable supply of raw materials and growing public awareness for green business operations. Replacing fossil fuels with a renewable source like biomass and it's derived feedstock is therefore considered a potential alternative that may provide a way to meet the demand (Bardhan et al. 2015). In this regard, many reviews have highlighted the importance of biomass conversion technologies to produce biorenewable chemicals (Dutta et al. 2012; Tuck et al. 2012; van Putten et al. 2013; Schwartz et al. 2014; Bardhan et al. 2015). In 2004, the US Department of Energy (DOE) has identified top 12 platform molecules that can be produced from biomass via biological or chemical processes (Werpy and Petersen 2004). While most of these platform molecules such as 5-hydroxymethyl furfural (HMF), furfural, levulinic acid (LA) and gamma-valerolactone (GVL) are synthesized by chemical methods (Alam et al. 2012, 2014; Alam and Saha 2015), some are produced via fermentation (Alam et al. 2015) as shown in Fig. 1. Later on, in 2010, a revision was made to add new platform molecules of industrial importance (Bozell and Petersen 2010). Recent research work has introduced the potential of additional biomass-derived molecules such as terpenoids and 2-pyrones (Fig. 1) which may have diverse applications in medicines, pesticides, fuels and chemicals

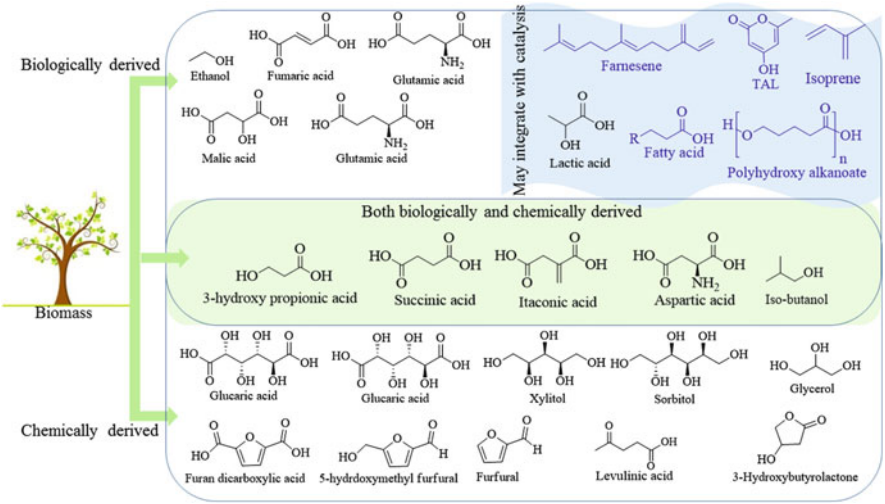

Fig. 1. List of top chemicals produced from biomass. Blue-coloured molecules are not listed in DOE list but have shown a significant potential as platform chemicals in past few years

(Chia et al. 2012b; Becker and Wittmann 2015). Due to structural complexity, some of these molecules are difficult to synthesize chemically in large quantity. Therefore, industrial production relies on extraction from biological sources (Ghaffar et al. 2014). Bioprocessing methods can be further distributed into two classes based upon the use of specific microorganism or an enzyme (Welsh et al. 1989; Straathof 2014). The microbiological methods may further be subdivided into biosynthetic and biotransformation approaches. Biosynthesis is the process of achieving the desired yield of a product by engineering metabolic pathways (Becker and Wittmann 2015). Biotransformation, on the other hand, involves the use of microbial cells to perform specific modifications of chemical structures.

Herein, key biological pathways for making selective chemicals from biomass are discussed. The intermediates can potentially be converted into high-value products and advanced biofuels via fermentation or an integrated bio and chemocatalytic processing. Figure 2 shows a summary of the metabolic pathways for the production of chemicals and platform molecules such as alcohols, fatty acids, isoprenoids, lactones and pyrones. These pathways can be integrated to a chemical transformation step to yield desired product. For example, fatty acid and its derivatives are biologically produced by the yeast, *Saccharomyces cerevisiae*, and bacteria, *Escherichia coli*, in high concentrations. Fatty acids may find applications in the making of detergents, cosmetics, soaps, lubricants and pharmaceuticals (Zhou et al. 2014). Through chemical catalysis routes such as decarboxylation, decarbonylation and esterification reactions, fatty acids may be converted into alkenes, alkanes and fatty acid methyl esters (FAMEs) (Lennen et al. 2010; Na et al. 2010). In order to design an efficient biological route for the production of a desired chemical or a platform molecule, several factors may be considered which include microbial physiology, metabolism and the mechanism of the reaction catalyzed by enzymes (Hideaki and Sakayu 1988). While microbial physiology and metabolism are concerned with investigation on factors effecting the growth and metabolism, enzyme catalysis focuses on studying the properties and mechanism of enzymatic reactions and the overall evaluation of a specific enzyme as a potential catalyst to synthesize the molecule of interest (Koeller and Wong 2001). This chapter therefore focuses on discussing the key biological pathways with an emphasis on metabolic engineering approaches and consequent challenges for optimizing the production of desired products and intermediates for integrated processing.

2 Metabolic Pathways for the Production of Platform Molecules and Chemicals

The ability to engineer a metabolic pathway in a microorganism has shown new ways to obtain the desired product yield. The precision in the application of molecular level tools combined with increasing information on bioresources like microbial strains and genes and computing methods have simplified the study of

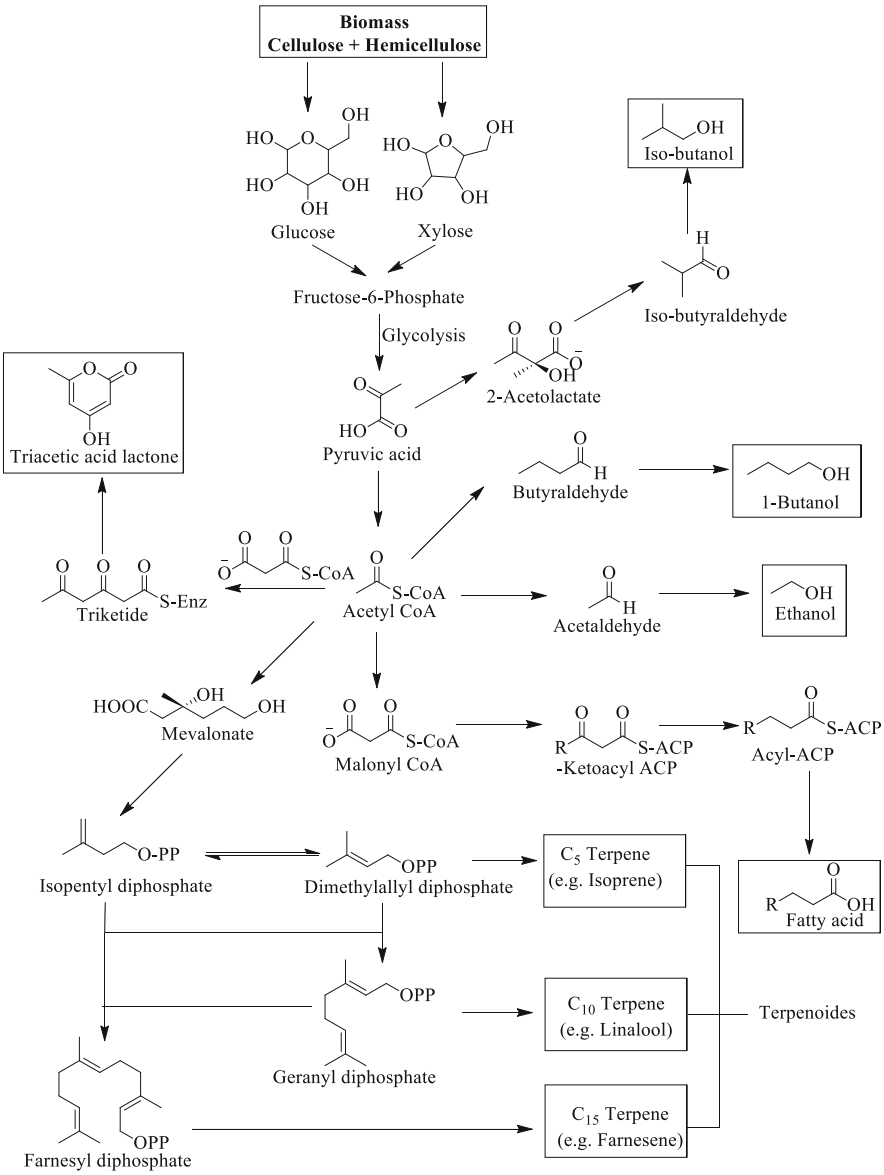

Fig. 2. Metabolic pathways for the synthesis of bio-based platform chemicals. Glycolysis is a direct route to covert glucose to pyruvate. Only major routes and products are shown here without indicating the formation or consumption of other molecules or side products

complex biological routes to produce advanced fuels and value-added chemicals by engineered microorganisms. The products of interest could be a primary or secondary metabolite. The primary metabolites are the products whose production is associated with growth (ethanol, lactic acid, etc.), while secondary metabolite refers

to compounds produced late in the growth or whose synthesis occurs after the growth is ceased (e.g. triacetic acid lactone). Fatty acid is a typical example of primary metabolite that is synthesized in the cytoplasm of the cell, in the form of phospholipids and triglycerides (Brown et al. 1996). The fatty acid is synthesized by fatty acid synthase (FAS) enzyme, utilizing acetyl-CoA via the synthesis of malonyl-CoA (Fig. 2) (Zhou et al. 2014). A number of yeast and bacterial species have been reported for fatty acid synthesis (Hiltunen et al. 2009). Depending upon the location of synthesis and application, the metabolic pathway reported in yeast (e.g. *S. cerevisiae*) has been observed to be different from bacteria (e.g. *E. coli*) (Zhou et al. 2014). FAS could be regulated by engineering the metabolic pathways (Beld et al. 2015). For example, on developing a control on the production of malonyl-CoA in genetically engineered *E. coli*, an effective redirection of carbon flux towards fatty acid was obtained, resulting into the regulation of FAS (Tee et al. 2014). The controlled pathway, therefore, showed a 15.7-fold improvement in the yield of fatty acid as compared to the wild-type strains (Xu et al. 2014).

In addition to microbial processes, platform molecules can be synthesized by enzymatic materials, involving extracellular or intracellular processes. The three-dimensional nature of the active site in the enzyme helps in the selective conversion of substrate molecule (Neurock 2010). Cellulolytic and hemicellulolytic enzymes act specifically to deconstruct cellulose and hemicellulose into their individual sugar monomers. The C_6 and C_5 sugars thus obtained can be selectively converted into a desired molecule by utilizing a range of available biological routes. Several metabolic pathways act selectively on a substrate, and the choice of pathway is dictated by the type of microorganism used (Peralta-Yahya et al. 2012; Straathof 2014). Consequently, genetic engineering techniques may help in preventing side reaction products in a metabolic pathway and thus improving the overall yield and selectivity.

Metabolic routes involving polyketide synthase (PKS) are experimented to produce a wide array of compounds such as fatty acids, chalcones and stilbenes. Utilizing this pathway, a diverse range of bacterial and fungal secondary metabolites such as macrolide antibiotics, lovastatin, etc., are produced (Khosla and Pfeifer 2001). PKS is known to yield triacetic acid lactone (TAL), which has been recently suggested as a potential platform molecule to produce biorenewable chemicals (Chia et al. 2012a, 2013). On overexpressing the 2-pyrone synthase gene in *Saccharomyces cerevisiae*, the yield of TAL was increased by 37-fold to a titre of 2.2 g/l (Cardenas and Da Silva 2014). Thus, the metabolic engineering techniques can be successfully applied in PKS to achieve a desired yield of the product. Interestingly, PKS offers a unique ability to selectively produce alkyl chains with varying length (C_4–C_{30}) and more based upon the starter and extender units utilized in the synthesis (Nikolau et al. 2008; Yu et al. 2012). For instance, methyl triacetic acid lactone (MTAL) having methyl group at C_3 is produced biologically from fermentation of glucose by *Penicillium stipitatum* using acetoacetyl-CoA and methyl malonyl-CoA as the starter and the extender units (Funa et al. 2002) as shown in Fig. 3, respectively. Similarly, formation of tetra-acetic acid lactone (TAAL) is reported from the same metabolic route of TAL, wherein 2 moles of

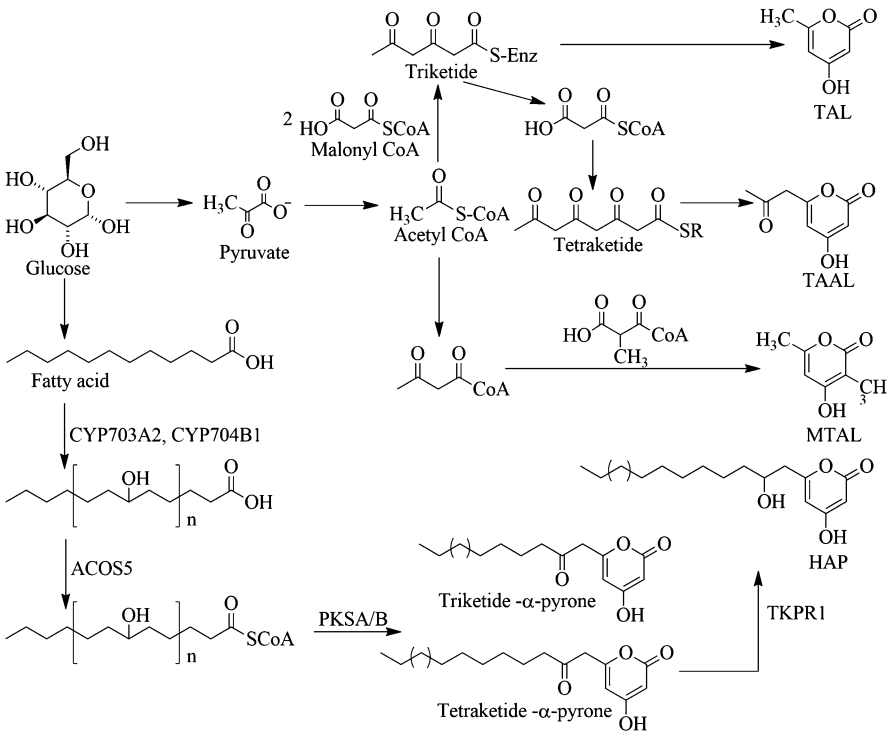

Fig. 3. Metabolic routes showing glucose conversion into 2-pyrones. Only major products are shown here without indicating the formation or consumption of other molecules or side products

extender malonyl-CoA are utilized with the starter acetyl-CoA (Bentley and Zwitkowits 1967) as shown in Fig. 3. Alkyl-α-pyrones with varying chain length at the C_6 position have been produced via the synthesis of fatty acid and its subsequent conversion by PKS. The routes for the biological synthesis of TAL, MTAL, TAAL, tri-and tetraketide α-pyrone including hydroxyl alkyl-α-pyrone (HAP) are shown in Fig. 3.

Some of the key structural features of 2-pyrones, e.g. the position of the double bond in the ring and the type and position of the substituent, were observed to directly correlate to the yield of a desired product. Interestingly, TAL was observed to ring open and decarboxylate at unprecedented low temperatures (<100 °C) with high conversions (>99%) and selectivity (>99%) in water, without the requirement of a catalyst (Chia et al. 2013). It was demonstrated by ab initio DFT simulations, that in aqueous systems, partially saturated 2-pyrone molecules undergo direct ring opening and decarboxylation via a retro-Diels-Alder (rDA) reaction, in which water helps in stabilizing the zwitterionic intermediate (Gupta et al. 2016; Khan et al. 2016). TAL could therefore act as a potential precursor to produce important chemicals such as sorbic acid, bioactive molecules (e.g. phloroglucinol, which deoxygenates to form 1,2,3,4-tetrahydroxybenzene and hydroxyhydroquinone),

fuel additives or fuels such as 2,4-hexadienoic acid, 1,3-pentadiene, etc. (Hansen and Frost 2002; Chia et al. 2012b, 2013). Similarly, a fermentation derived molecule, 6-amyl-2-pyrone can yielded C9 ketones and C14/C15 hydrocarbon fuels (Alam et al. 2016).

Compared to pyrones, saturated lactone molecules may require high temperature and pressure conditions for ring opening and decarboxylation, which constitutes a key transformation step. For example, the ring opening and decarboxylation of γ-valerolactone (GVL) proceeds on a solid acid catalyst (SiO_2/Al_2O_3) at a relatively higher temperature (>300 °C) and pressure (>30 bar) conditions as compared to TAL. Nevertheless, GVL is an important platform molecule, and the product obtained after ring opening and decarboxylation, 1-butene, may be used for synthesizing diesel rage fuels (Bond et al. 2011). Similar to GVL a group of lactone molecules (Shi et al. 2015) such as γ-butyrolactone (GBL), γ-caprolactone (GCL), δ-valerolactone (DVL) and ε-caprolactone (ECL) can be produced from biomass or it's derived sugars via fermentation or chemical catalytic transformations as shown in Fig. 4 (Isikgor and Becer 2015). Chia et al. have shown the synthesis of GCL from biologically synthesized TAL in a series of chemical transformation including hydrogenation and ring-opening reactions (Chia et al. 2013). Metabolic pathway engineering may be utilized to optimize lactone yield in fermentation. Polyhydroxyalkanoate synthesis routes have been shown to produce DVL as shown in Fig. 4 (Van Walsem et al. 2012; Zhuang et al. 2014). High titre of GBL was reported to be produced in glucose fermentation by genetically engineered

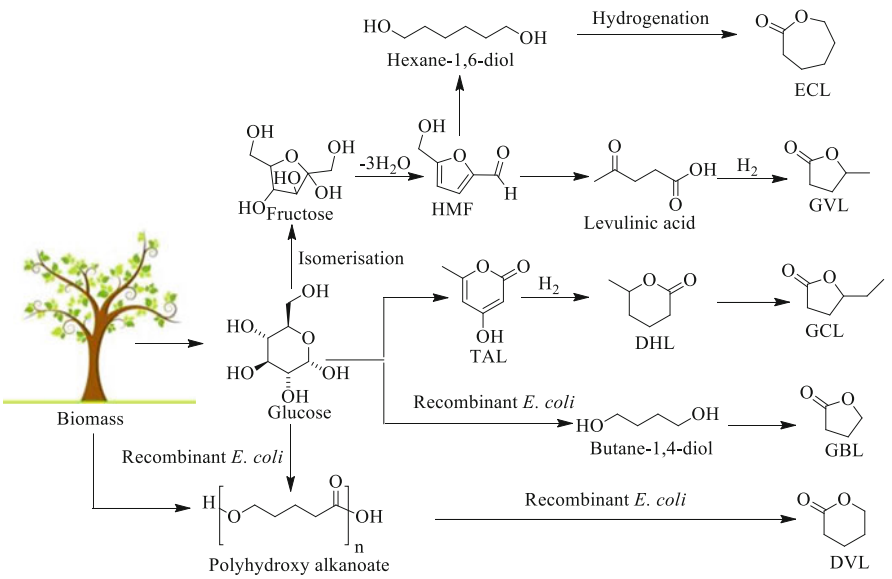

Fig. 4. Synthetic routes for biomass-derived lactone molecules: ε-caprolactone (ECL), γ-valerolactone (GVL), γ-caprolactone (GCL), γ -butyrolactone (GBL) and δ-valerolactone (DVL) [adapted from Gupta et al. (2016)]

E. coli (Hwang et al. 2011; Yim et al. 2011). In a separate route, GBL could also be produced as a major side product by recombinant *E. coli* in the synthesis of butane-1,4-diol. Similarly, concentration up to 15.7 g/l of ε-caprolactone (ECL) was obtained by Baeyer-Villiger oxidation of cyclohexanone using recombinant *E.coli* (Lee et al. 2014).

The reactivity of lactones towards catalytic ring-opening reaction plays an important role in their transformation to a commodity product. Density functional theory (DFT) calculations suggest that the size of the ring and the substituent of the lactone molecules are important structural parameters, which ascertain it's reactivity (Gupta et al. 2016). A stable oxocarbenium ion was suggested to form in the aqueous phase (Amyes and Jencks 1989). The oxocarbenium ion formation energy in the gas phase was computed for the lactones with increasing ring size, and it was observed to correlate well to the reactivity of the ring-opening step. Biomass-derived lactones may be used for obtaining a variety of products. For example, GBL can be used as a precursor for commodity and specialty products such as 1,4-butanediol (BDO), tetrahydrofuran (THF), *N*-methylpyrrolidone (NMP), *N*-ethylpyrrolidone (NEP), *N*-vinylpyrrolidone, etc. (Van Walsem et al. 2015).

Another interesting chemical group is alcohol, which has wide range of applications as solvents and starting molecules for synthetic organic chemistry. Amongst the biologically synthesized alcohol products, ethanol, propanediol and butanediol are important to be discussed here. Propanediol itself is used as de-icer, antifreeze, moisturizer, food additive and a monomer for polymer production. Butanediol is suggested to be a platform molecule yielding commodity products such as drugs, cosmetics and solvents (Becker and Wittmann 2015). Fermentation of glucose under aerobic and anaerobic condition produces ethanol which is a direct fuel additive. In anaerobic condition, yeast produces ethanol from pyruvate which is derived from glucose in a two-step conversion process. On decarboxylation of pyruvate, acetaldehyde is formed which is subsequently reduced to ethanol by alcohol dehydrogenase as shown in Fig. 2. Similar to ethanol, 2,3-butanediol is produced from pyruvate via the formation of acetolactate and acetoin.

Recent interests in research is directed towards microbial production of long chain alcohols, which have been shown to possess better fuel properties compared to ethanol (Nielsen et al. 2013). Most recently, Basen et al. have applied metabolic engineering to hyperthermophile *Pyrococcus furiosus* to produce a range of alcohols including ethanol (Basen et al. 2014). The engineered strain converts glucose to ethanol via the formation of acetate and acetaldehyde, catalyzed by aldehyde ferredoxin oxidoreductase (AOR) via heterologous expression of bacterial alcohol dehydrogenase gene. Similarly genetically engineered *Pyrococcus furiosus* strain was observed to successfully produce propanol, isobutanol, 1-pentanol, isoamylalcohol, 1-hexanol and phenylethanol from propionate, isobutyrate, valerate, isovalerate, caproate and phenylacetate, respectively (Basen et al. 2014).

Terpenoids or isoprenoids derived from isoprene units are naturally produced in plant which on organic extraction could be used for various applications such as in pharmaceutical and fuel synthesis. Fluctuations in prices and limited availability of such natural products, however, limit their applicability. Metabolic engineering techniques have been applied for heterologous expression of mevalonate (MVA)

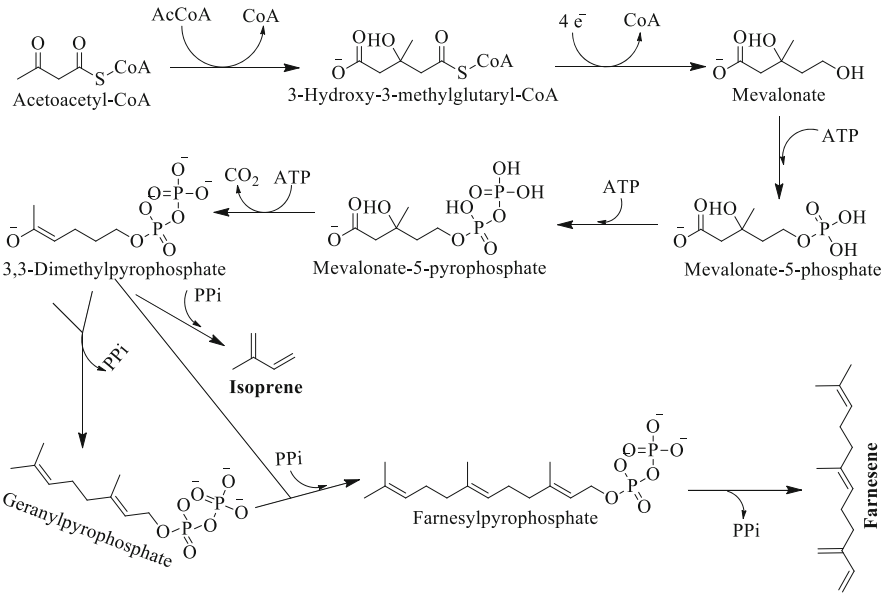

Fig. 5. Pathway showing isoprene and farnesene biosynthesis. PPi is pyrophosphate [adapted with permission from Straathof (2014). Copyright © 2016, American Chemical Society]

metabolic pathway to build two basic C_5 units of terpenoids, namely, dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) as shown in Fig. 2. The resultant condensation product of these two units lead to the formation of C_{10} and C_{15} terpenes, which are generally termed as di- and triterpenoides, respectively. Isoprene is the monoterpene, which has potential application as polymer precursor to produce synthetic rubber for manufacturing various products such as baby toys, medical equipments, tyres, etc. In MVA pathway, isoprene is produced by the elimination of pyrophosphate (PPi) from 3,3-dimethylallyl pyrophosphate utilizing the enzyme isoprene synthase (Fig. 5). Similarly, farnesene (C15 terpenoid) can also be synthesized via MVA pathway in a series of reactions as shown in Fig. 5. Upon hydrogenation, farnesene is converted into farnesane, a fuel having diesel properties which is being explored by Amyris Inc. Emeryville, USA, in a demonstration plant (Peralta-Yahya et al. 2012). Isoprene is naturally produced by algae, plants, bacteria and animals. The production of isoprene is reported through heterologous expression of the gene encoding isoprene synthase (*IspS*) in a photosynthetic *Cyanobacteria* (Lindberg et al. 2010). Recent study by Yang et al. have observed enhanced yield (6.3 g/l after 40 h of fed-batch fermentation) of isoprene on optimizing the MVA pathway in *Enterococcus faecalis* by introducing mutation in the *mvaS* gene (Yang et al. 2012). Although these results are encouraging, from a commercial perspective, it may not be economical to produce fuel range compounds at this stage of productivity.

3 Challenges in Biological Processing

Biological processes provide wide range of opportunities for chemical and biochemical engineers in the development of new synthesis methods for large-scale production of intermediate chemicals, fuels and pharmaceuticals. However, limitations, such as inability to utilize abundant and inexpensive substrates by the microbial enzymes, hamper the yield and selectivity of the desired product (Bailey 1995). Majority of the microorganisms employed do not possess enzymatic capability to utilize widely available and cheaper carbon sources like agricultural or food processing waste. To utilize such feedstocks, enzymes having potential for degradation and uptake are required. Genetic level manipulations therefore offer a promising approach to overcome their limitations.

Insufficient supply of a rate or yield-limiting resource is another challenge that requires improvement. Certain feedstock sources have comparatively low solubility, and they may exist as a heterogeneous phase in the bioreactor. The processes employed therefore may require enhanced mixing, multiphase contacting schemes and efficient dispersion system to increase the rate of substrate transport. Inhibitory actions on microorganisms by microbial metabolism or produced chemicals are another concern that requires continuous separation or elimination of product from fermentation media. Novel approaches involving in situ product extraction in bioreactors employing biphasic medium, as in the case of hexanoic acid production, have shown considerable promise (Choi et al. 2013). Genetic manipulation to overcome this problem is rather difficult in view of the poorly understood inhibition mechanism. Another important challenge is inadequate utilization of substrate that leads to higher operating cost (Li et al. 2014). Production of chemicals (e.g. alcohol) at higher temperature (>70 °C) is beneficial in terms of lower risk of microbial contamination, higher diffusion rates and lower cooling and distillation expenses. However, very few microorganisms are reported, which are capable to grow and generate alcohol at temperature higher than the ambient (Svetlitchnyi et al. 2013). An archaebacteria, *Pyrococcus furiosus*, is therefore investigated that is capable enough to produce a range of lower and higher-range alcohols, which grow optimally near 100 °C (Basen et al. 2014). Similar advancement in the identification and metabolic engineering of microorganism is therefore required to produce other products of interest.

4 Concluding Remarks

Most of the chemical processes require harsh reaction condition consuming high energy inputs that may cause harm to environment. While existing biological routes provide a potential alternative choice by offering several advantages. However, issues persistent in the biological process hamper yield and selectivity of the desired products. Noteworthy of them are (a) incompatibility with large volume and

inexpensive substrates, (b) insufficient transported supply of a rate-limiting or yield-limiting substrate, (c) inhibition due to excess substrate, (d) inhibition by the product resulting in low effluent product concentration and (e) inadequate rate of formation or yield of the desired product. Improved biological routes are therefore being explored to address aforementioned concerns that favour processes which can run under milder condition. Interestingly, by applying metabolic engineering techniques, the microorganisms may be transformed with tremendous potential to induce fresh enzyme systems capable of utilizing new substrates into useful products. Furthermore, it could be possible to obtain and cultivate microorganisms susceptible to temperature, acids and alkalis, which are capable of producing unique enzymes stable towards heat, alkali or acids. With the advancement in cultivation and enzyme purification techniques along with genetic and protein engineering, the special functions of particular microorganisms are enhanced. These methods can further be integrated to a suitable catalytic transformation step to yield new product of interest. In this regard, successful biological synthesis of certain groups of molecules such as terpenoids and 2-pyrones may open up avenues for the development of novel integrated processes for the synthesis of pharmaceuticals, fine chemicals, fuels and precursors for polymers.

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Part III
Industry

Green Synthesis of Hydroxamic Acid and Its Potential Industrial Applications

Bhatia Ravi Kant, Bhatia Shashi Kant, Bhalla Tek Chand, and Bhatt Arvind Kumar

Abstract Hydroxamic acids are the derivatives of hydroxylamine and carboxylic acid. These produce stable chelates with metal ions and are important constituents of several useful compounds. Generally, hydroxamic acids are synthesized through chemical route by N-alkylation of simple O-substituted hydroxylamine with a variety of alkylating agents. However, large-scale synthesis of hydroxamic acids by chemical route is not only expensive, but the product also contains several by-products as impurities. In the last two decades, several reports on enzymatic synthesis of hydroxamic acid using lipase, nitrilase, and amidase have been published. Enzyme-mediated synthesis of hydroxamic acid produces pure products even under mild conditions, specially temperature and pH. Hydroxamic acids find application as insecticides, antifungal agents, antimicrobials, siderophores and plant growth regulators, anti-HIV, antimalarial, antineoplastic agents, tumor suppressers, MMP inhibitors, and other robust applications in health care. These are also used in nuclear technology and wastewater treatment. Enzyme-mediated synthesis of hydroxamic acids and their various possible applications have been discussed in this chapter.

Keywords Amides • Hydroxylamine • Acyl transfer activity • Hydroxamic acid

1 Introduction

Hydroxamic acids are one of the most extensively studied chemical compounds due to their tremendous applications in various fields. These are a class of chemicals in which a hydroxylamine is inserted into a carboxylic acid. Structure of hydroxamic

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acid is represented as R–CO–NH–OH, where R is an organic residue, CO is a carbonyl group, and hydroxylamine as NH_2–OH (Agarwal and Kunji 2005). Iron is an important component of cytochromes and Fe–S proteins. Hydroxamates are important growth stimulators for different microorganisms which being iron-binding compounds help in the movement of iron molecules inside the microbial cells. These iron–sulfur protein compounds are very essential in cellular respiration. Under oxic conditions, iron is present in the form of minerals, i.e., Fe^{3+} oxidation state. Hydroxamic acids are very strong chelating agents, these are secreted by bacteria. Firstly these form an iron–hydroxamate complex, once they formed and entered into the cell, subsequently the iron molecule is removed from this complex by various enzymatic reactions. Then this iron is used up by cell in different metabolic activities and building blocks of cell, and hydroxamic acid is excreted outside the cell to uptake another iron molecule.

About more than 100 years ago, Lossen discovered the hydroxamic acids, and since then a huge amount of research work has been carried out on the structure, synthesis, and structure–activity relationships (SAR) and applications of hydroxamic acids (Ragno et al. 2004; Mai et al. 2005). In biomedical sciences, hydroxamic acid moieties are utilized to target various types of ailments like cancer, cardiovascular diseases, HIV, Alzheimer, malaria, allergic diseases, tuberculosis, etc., which is successfully achieved by inhibiting the activity of enzymes such as urease and matrix metalloproteinases (Hara et al. 2000; Jamal and Pang 2000; Leung et al. 2000; Valapour et al. 2002; Munster et al. 2001; Mishra et al. 2002). These compounds and their derivatives on one hand were used as antioxidants, as corrosion inhibitors, and for extraction of toxic elements and rare earth metals and on other hand also serve as redox switches for various electronic devices (Thorarensen et al. 2001; Kalman et al. 2000; Amit et al. 2001; Taira et al. 2002). Production of hydroxamic acids by enzymatic routes and their various potential applications have been discussed in this chapter.

2 Types of Hydroxamic Acids

Various types of hydroxamic acids have been reported so far by various researchers in the past. Different hydroxamic acids have different properties which are due to the type of acyl group and the length of carbon chain. Depending upon the length of carbon chain, hydroxamic acids can be classified as follows:

(a) **Short-chain hydroxamic acids**
Short-chain hydroxamic acids have a carbon chain of C2–C3. These are easily soluble in water, but some of the hydroxamic acids with very high chelating properties are used in resin synthesis, e.g., acetohydroxamic acid and propionohydroxamic acid (Sharma et al. 2012).

(b) **Middle-chain hydroxamic acids**
Middle-chain hydroxamic acids have carbon chain of C4–C8. Generally these are aminohydroxamic acids. These are very useful in medical field since they are used to inhibit various metalloproteases. Potential applications of hydroxamic acids, such as butyrohydroxamic acid, valerohydroxamic acid, succinyl hydroxamic acid, benzohydroxamic acid, nicotinyl hydroxamic acid, homocysteine hydroxamic acid, suberoylanilide hydroxamic acid (SAHA), etc., have been well established in various processes (Celine and Kelvin 2000).

(c) **Fatty hydroxamic acids or long-chain hydroxamic acids**
These are generally long carbon chains of more than C12. These are insoluble in water and are very useful for removing toxic metal ions present in water. These are used as surfactants in detergent industry and also exhibit antibacterial and antifungal activities, e.g., actinonin, mycobactin, phenylalanyl hydroxamic acid, lauryl hydroxamic acid, palmityl hydroxamic acid, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), etc. (Suhendra et al. 2005; Haron et al. 2012).

3 Synthesis of Hydroxamic Acid

Hydroxamic acid can be synthesized both by chemical and by enzymatic processes which are as follows:

Chemical Method
Chemically, hydroxamic acids can be synthesized by a number of reactions involving acids, esters, aldehydes, etc., detailed below.

From Acids
Different types of carboxylic acids have been successfully used to synthesize variety of hydroxamic acids. Since Loosen arrangement is one of the key methods for their synthesis, hence, ultrasonication is done for the accelerated production of these valuable acids (Vasantha et al. 2010).

From Esters
Ethyl or methyl carboxylic esters can be converted into the corresponding hydroxamic acids. Hydroxylamine when mixed with esters in the presence of a base under suitable conditions produced hydroxamic acid in good quantity. This method has been frequently used for their production without loss of their stereochemical integrity (Massaro et al. 2007; Riva et al. 2009).

From Aldehydes
Amidation is one of the important methods for the production of hydroxamic acids. In this reaction hydroxylamine reacts with aryl/alkyl/alkenyl/heterocyclic aldehydes in the presence of nitroso compounds for the synthesis of respective hydroxamic acids. This chemistry has also been extended to a three-component reaction for the synthesis of *N*-arylaziridines (Wong et al. 2008).

Hydroxamic Acid by Green Chemistry, i.e., Biocatalyst

Hydroxamic acid can also be synthesized by using biocatalysts (enzyme) mainly by those enzymes which are capable of transferring an acyl moiety from one compound to another. Enzymatic synthesis of hydroxamic acid requires two substrates, i.e., acyl group containing acid/amide/ester/hydrazide/fatty acids and highly nucleophilic hydroxylamine on which the particular acyl group can be transferred. Enzymatically, hydroxamic acid is synthesized by the use of three enzymes, i.e., lipase, nitrilase, and amidase. Out of these three enzymes, amidases have been extensively used for the production of variety of hydroxamic acids.

Lipase

Lipids and fats are catalyzed to corresponding glycerols and fatty acids by lipase enzyme. Most of lipases come under the category of esterase. These enzymes having EC 3.1.1.3 are also called as triacylglycerol hydrolases. Generally, a biphasic medium is required for the synthesis of fatty hydroxamic acids. Production of these types of hydroxamic acids requires two substrates as acyl donors, i.e., fatty acid or fatty acid methyl ester and hydroxylamine. The acyl transfer activity of this enzyme helps to transfer the acyl group from hydroxylamine to fatty acids and transform them to corresponding fatty hydroxamic acids (Jahangirian et al. 2011).

Nitrilase

Nitrilases, E.C 3.5.5.1, break the (–C≡N) triple bond of nitriles to form carboxylic acids. Besides biotransformation of nitriles, this enzyme also catalyzes the synthesis of hydroxamic acids. Nitrilases exhibit dual activity, i.e., hydrolytic activity in aqueous reaction medium and acyl transfer activity in the presence of more nucleophilic hydroxylamine (Dadd et al. 2001). Acyl transfer activity of nitrilase is involved in the transfer of acyl group from nitriles or amides to hydroxylamine to form hydroxamic acid (Vejvoda et al. 2011).

Amidase

Amidase (E.C 3.5.1.4) is one of the unique enzymes in the living world. These versatile enzymes with broad substrate specificity convert amides to the corresponding carboxylic acids and ammonia. Apart from the amide degradation, these also catalyze the transfer of an acyl group of different amides to hydroxylamine to produce the corresponding hydroxamic acids (Fournand and Arnaud 2001; Bhatia et al. 2013) as shown in Fig. 1. This enzyme is a very important tool for the synthesis of hydroxamic acids as well as for various industrially significant compounds.

4 Mechanism of Enzymatic Synthesis of Hydroxamic Acid

Amidase exhibit “Bi-bi Ping-Pong” mechanism for the acyl transfer activity. Amides first react with the enzyme to give acyl-enzyme complexes (E–S complexes) which subsequently leads to formation of carboxylic acids. If a strong

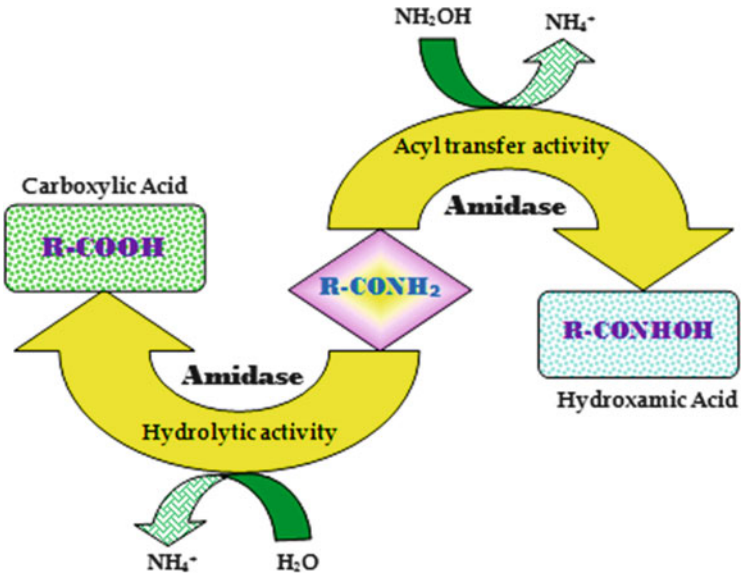


Fig. 1 Types of reactions catalyzed by amidases (Bhatia et al. 2013)

nucleophilic agent like hydroxylamine is present instead of water (in case of acyl transfer activity) then its interaction with E–S complex results in the production hydroxamic acids. After the formation of the product, the enzyme retains its original state and is ready to convert another molecule of amide and hydroxylamine to hydroxamic acid (Pandey et al. 2011; Sharma et al. 2012).

5 Possible Action Mechanism of Hydroxamic Acid Against Metalloenzymes

Hydroxamic acids contain a metal-binding moiety (–NHOH). The typical structure of hydroxamic acids has three different parts: (i) a strong metal ion chelating group, (ii) a long aliphatic or aromatic carbon chain which acts as a linker, and (iii) a capping group. All these parts collectively interact with different groups present in the pocket of active site of metalloenzyme (Chen et al. 2011). When hydroxamic acid enters into the catalytic site of hydroxamic acid, it competes with the substrate molecules, and due to its strong chelating property, it binds faster than substrate and finally inhibits the activity of these enzymes (Belvedere et al. 2005) as shown in Fig. 2. The hydroxamic acids such as abexinostat, belinostat, panobinostat, dacinostat, givinostat, pracinostat, resminostat, and vorinostat directly inhibit the metalloenzymes by binding to its active site (Marks and Breslow 2007; Codd et al. 2009). A large number of clinical trials have been carried out using hydroxamic acids in patients with different ailments. Among various hydroxamic acids reported

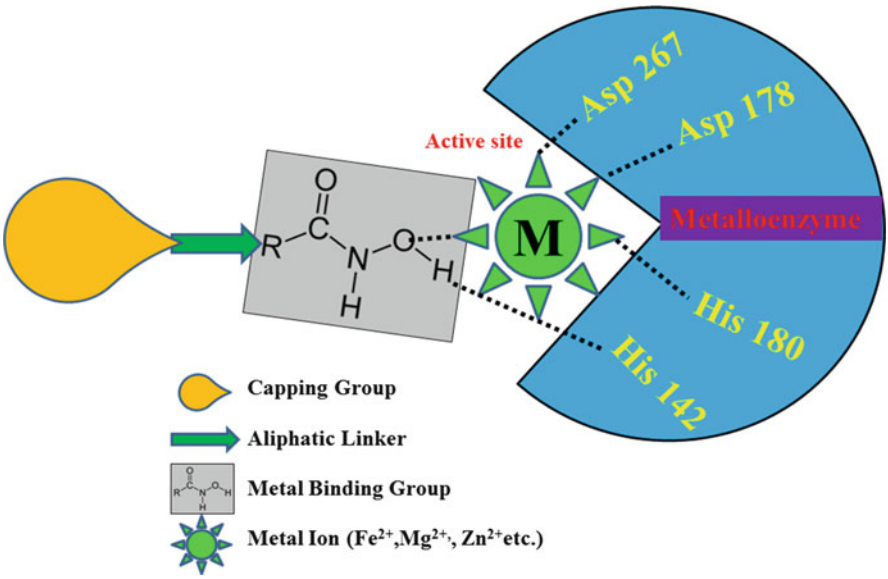


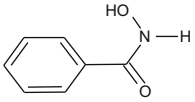
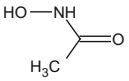
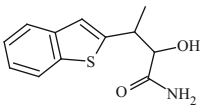
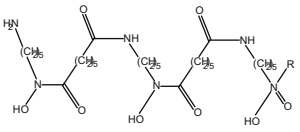
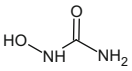
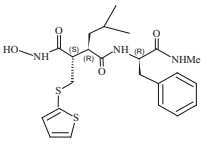
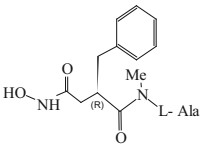
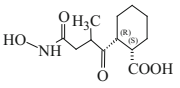
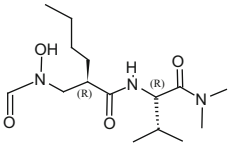
Fig. 2 General structure of a hydroxamic acid and its interaction with the metalloenzyme

so far, belinostat, panobinostat, pracinostat, abexinostat, dacinostat, and resminostat are under clinical trials in phase I and phase II for cancer treatment (Federico and Bagella 2011; Ververis et al. 2013). Due to the huge successes rate of vorinostat, the US Food and Drug Administration (FDA) has proved it for the treatment of different malignancies (Lemoine and Younes 2010; Howman and Prince 2011; Grant et al. 2007).

6 Applications of Hydroxamic Acids

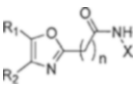
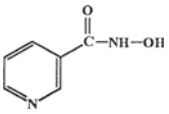
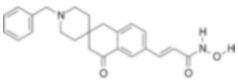
A number of metalloenzymes, such as histone deacetylase, matrix metalloprotease, lepoxyginase, urease, angiostatin, leukotriene A4 hydrolase, carbonic anhydrase, etc., have been reported to accelerate cell division and thus resulting into cancer (Marks et al. 2001). If the activity of these enzymes could be blocked by the use of some metal-scavenging compounds, then a prevention strategy can be evolved for such ailments. Some of the hydroxamic acids such as vorinostat, belinostat, panobinostat, trichostatin-A, and histone deacetylase (HDAC) inhibitors have anticancerous properties (Richon 2006; Yue et al. 2015). These hydroxamic acids are expected to be available in the market after their successful clinical trials. A detailed list of various hydroxamic acids along with their potential applications has been given in Table 1.

Table 1 Applications of some important hydroxamic acids

Hydroxamic acids	Structure	Uses	Reference
Benzohydroxamic acid		Antitumor, antineoplastic	Bhatia et al. (2012)
Acetohydroxamic acid (Lithostat)		To treat ureaplasma, anemia, anti-HIV agent	Pandey et al. (2011)
Fatty hydroxamic acids		Anti-inflammatory **To treat chronic asthma**	Haron et al. (2012)
Deferoxamine B (Desferal)		Antimalarial	Giannini et al. 2015)
α-Aminohydroxamic acid		Anti-HIV agent, Psoriasis inhibitor	Munster et al. (2001)
Marimastat		To treat small cell lung cancers	Muri et al. (2002)
Inhibitor of LTA_4		Anti-inflammatory	
Idrapril		Render cardioprotective effects	
N-formyl hydroxylamine BB-3497		Antibacterial agent	
Cyclic hydroxamic acids	2,4-Dihydroxy-1,4-benzoxazin-3-one (DIBOA) 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)	Provide resistance against pathogen and insects	Copaj et al. (2006)

(continued)

Table 1 (continued)

Hydroxamic acids	Structure	Uses	Reference
Unsaturated and middle-chain hydroxamic acid		Wastewater treatment Nuclear technology	Haron et al. (2012)
Nicotinyl hydroxamic acid		Tyrosinase and melanin inhibitor	Chen et al. (2011) Bhatia et al. (2014)
Spiropiperidine hydroxamic acid		HDAC inhibitor	Varasi et al. (2011) Vreese et al. (2015)
Suberoylanilide hydroxamic acid (SAHA)		Anticancerous	Bosiack et al. (2011)
Long-chain hydroxamic acids		As surfactants in detergent industry	Jahangirian et al. (2011)
Poly(hydroxamic acid)		Used for gravimetric analysis and scavenging of heavy metal ions	Hassan et al. (2011)

7 Hydroxamic Acid as Histone Deacetylase Inhibitor

Histone deacetylase (EC 3.5.1.98, HDAC) are a class of enzymes that eliminate the acetyl groups from the histone proteins having an ε-*N*-acetyl lysine amino acid. This removal of acetyl group allowed DNA strand to wrap histone more tightly. The tight wrapping of histone by DNA regulates acetylation and deacetylation of this protein which finally controls the overall expression of DNA. Any change in the expression and mutations in the gene of HDACs leads to development of tumor due to uncontrolled cell proliferation, cell cycle, and apoptosis. HDACs are therefore one of the key targets for treating cancerous growth by developing potent HDAC inhibitors (Marks et al. 2001; Hanessian et al. 2007; Marks and Breslow 2007; Grassadonia et al. 2013; Giannini et al. 2015). These inhibitors block the active sites of HDAC and lead to aggregation of acetylated histones which finally suppress the tumor growth and inhibit cell proliferation and programmed cell death (Lu et al. 2005; Anandan et al. 2007).

8 Matrix Metalloproteinase Inhibitor

Matrix metalloproteinase (MMPs) are the enzymes that contain zinc ions in their pocket site. These enzymes are capable of removing the cell surface receptors, release of apoptotic ligands, and cytokine inactivation. Overexpression and activation of MMPs results in a number of ailments including arthritis, periodontal disease, multiple sclerosis, and metastasis that leads to various types of cancers. Hydroxamic acid-based MMP inhibitors strongly inhibit the activity of tumor cells and prevent their proliferation. Structure-based design suggests that hydroxamate-based MMP inhibitors suppress the metabolism and minimize the MMP activity (Van Lint and Libert 2007).

9 Treatment of Hypertension

Angiostatin-converting enzyme (ACE) is a type of integral membrane glycoprotein that plays an important role in regulation of blood pressure and electrolyte homoeostasis. Usually, ACE exists as membrane-bound enzyme, which is also present in blood plasma, amniotic fluid, seminal plasma, and other body fluids under normal conditions. Hypertension and a variety of other cardiovascular disorders are effectively cured by the use of ACE inhibitors. ACE inhibitors include ligands such as sulfhydryl (e.g., captopril), carboxyl (e.g., enalapril), phosphinyl (e.g., fosinopril), and more recently a hydroxamic acid group (e.g., idrapril). Hydroxamic acid and their derivatives that inhibit the MMP are very strong inhibitors of ACE (Apfel et al. 2000).

10 Treatment of Urinary Infection

Urease enzymes catalyze the breakdown of urea to ammonia and carbamic acid, which is further hydrolyzed to carbon dioxide and ammonia. This hydrolysis ultimately increases the pH and produces other ill effects on human and animal systems. Hydroxamic acids play a very significant role by inhibiting the urease activity. The mode of the inhibition involves coordination of the inhibitor with the nickel center in the enzyme. Acetohydroxamic acid binds to the active pocket of urease and inhibits its activity completely which is quite evident with SAR analysis. Based on this model, a number of heterocyclic hydroxamic acid derivatives have been designed and synthesized with greater binding stability than the dipeptide derivatives of hydroxamic acid (Muri et al. 2002).

11 Treatment of Allergic Reactions

The enzyme 5-lipoxygenase (5-LO) is one of the key enzymes for the synthesis of leukotrienes (LTs). 5-LO enzyme converts arachidonic acid into 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE), which is further converted to a series of highly potent leukotrienes by various metabolic reactions and finally triggers the allergic reactions. The peptidoleukotrienes like LTC4, LTD4, and LTE4 are slow-reacting substances of anaphylaxis. These peptidoleukotrienes are responsible for bronchial asthma, inflammation, tissue injury, liver diseases, and shock. Among various inhibitors, iron chelators (hydroxamic acid) play an important role in preventing the activation of LTs. These compounds were designed with the expectation that their functional groups might chelate iron and therefore inhibit the enzyme (Van Lint and Libert 2007).

12 Replacement of Nonsteroidal Anti-Inflammatory Drugs

The cyclooxygenase (COX) enzyme is responsible for the conversion of arachidonic acid into prostaglandins (PGs) that ultimately cause the inflammation. It has been found that COX-I inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the swelling after injury. But, NSAIDs also cause some side effects by inhibiting the COX-2 and may cause gastrointestinal bleeding and ulcer. So, in order to avoid the side effects of NSAIDs, there is an urgent need to develop new drugs that could inhibit only COX-1 and not COX-2 (Fiorucci et al. 2001). These findings stimulated several groups to design new potent molecules incorporating a hydroxamate moiety into the wide range of existing NSAID pharmacophores like hydroxamic acid and their derivatives of a known NSAID dibenzoxepine, isoxepac, etc. (Koncic et al. 2009).

13 Used Against Antibiotic-Resistant Bacteria

Peptide deformylase (PDF) is an important enzyme in microbial world which is believed to be playing a crucial role inside bacteria for the synthesis of cell wall and plasma membrane. PDF is also a very attractive target for drug design since it belongs to the family of metallohydrolases, one of the best studied enzymes (Wei et al. 2000). These enzymes require Fe^{2+} ion for their catalytic activity. Ferrous ion in PDF is bonded very loosely and hence can be easily oxidized into ferric ion, resulting in inactivation of enzyme. If ferrous ion is replaced with a nickel ion, then there is a little loss of catalytic power of enzyme and this can also enhance the stability of enzyme (Apfel et al. 2000). Since pathogenic strains are getting more and more resistant to the existing antibiotics day by day, hence, PDF can help to

provide a new target to destroy such bacteria (Apfel et al. 2000). Therefore, in order to develop new PDF inhibitor moieties, new strategies are developed and new chemical must be synthesized. PDF is a potent target for antibacterial drug design because (1) the gene linked with this activity is important to bacterial growth in vitro, (2) it is present in all bacteria, and (3) the enzyme's pocket has close resemblance with various metallohydrolases (Chen et al. 2000). Since PDF is a metallohydrolase, so hydroxamic acid is a potential inhibitor of this enzyme. Actinonin is a known hydroxamate-containing inhibitor of various metallohydrolases. The hydroxamate group of actinonin strongly acts as the chelating group that binds metal ion of the enzyme and inhibit its activity (Jayasekera et al. 2000; Wei et al. 2000).

14 Antibacterial Activity of Hydroxamic Acids

Hydroxamic acids are very essential component in defense mechanisms of several plants which function as natural pesticides. The cyclic hydroxamic acids DIBOA and DIMBOA play an important role in the defense of cereals against insects and pathogenic microorganism. Different *Erwinia* spp. spoiled maize crop by causing soft rot disease, but maize protects itself from this disease by secreting DIMBOA. A number of different hydroxamic acids exhibited similar antibacterial activity like DIMBOA against *Staphylococcus epidermidis*, *Enterococcus faecalis*, *P. aeruginosa*, and *Yersinia enterocolitica* (Pepeljnjak et al. 2005).

15 Insecticidal Property of Hydroxamic Acid

Hydroxamic acids have shown a lethal impact on the survival and reproduction of aphids. Different varieties of cereal like rye, wheat, and maize produce different types of hydroxamic acids that inhibit the growth of aphid *Metopolophium dirhodum*. It has been found that the survival rate of aphids fed with artificial DIMBOA was less than aphids fed with diets lacking DIMBOA. A similar relation between high hydroxamic acid levels in maize and resistance to the European corn borer *Ostrinia nubilalis* has also been reported (Copaj et al. 2006).

16 Hydroxamic Acid in Wastewater Treatment and Nuclear Technology

Hydroxamic acids have also been extensively investigated for their possible use in wastewater treatment and nuclear technology to evolve new methods to eliminate contaminating metal ions. This is a new but very promising approach to clean the wastewater contaminated with heavy metal ions (Haron et al. 2012).

17 Hydroxamic Acid in Analytical Chemistry and Detergent Industry

Hydroxamic acids play a very important role in analytical chemistry as reagents for gravimetric and spectrophotometric analysis of metal ions (Hassan et al. 2011). Besides their ability to form complex with metal ions, long-chain hydroxamic acids are also used as surfactants in the detergent industry (Jahangirian et al. 2011).

18 Conclusion

Hydroxamic acids the molecule of immense potential in pharmaceutical and other industries can be synthesized chemically as well as enzymatically. Enzymatic approach which results in production of pure hydroxamic acid without any side products can be used directly for medicinal purpose after successful clinical trials. Hydroxamic acids have metal chelating activity, and this property can be effectively used to treat diseases caused by various metalloenzymes. Various hydroxamic acids are used as anticancer, antibiotic, antibacterial, and anti-inflammatory agents and many more applications. The diversified activity profile of these compounds, i.e., if a particular molecule changing its locations within the same hydroxamic acids provides a new molecule which can be used for various potential applications in different industries. Therefore, it is the utmost need of time to search out new hydroxamic acid moieties with tremendous applications.

19 Opinion

A number of hydroxamic acids, viz., acetohydroxamic acid, butyrohydroxamic acid, benzohydroxamic acid (BHA), and succinic hydroxamic acid, have been synthesized using different types of biocatalysts. All these hydroxamic acids find application various fields. So there is a need to undertake studies with a focus to find or evolve more active, stable, and selective enzymes with acyl transfer activity to synthesize more potent hydroxamic acids with wider applications and without any by-product.

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Bioactive Natural Products: An Overview, with Particular Emphasis on Those Possessing Potential to Inhibit Microbial Quorum Sensing

Vijay Kothari, Pooja Patel, and Chinmayi Joshi

Abstract Bioactive natural products have formed the core of most ancient systems of healthcare and medicine. Crude natural preparations have been used for relief in a variety of infections and disease conditions. This review starts with a general description of the bioactive natural products, followed by the information on natural products being used for dealing with infectious microorganisms. In the latter section, much emphasis has been on the natural products capable of disrupting microbial communication, i.e., quorum sensing. Quorum sensing inhibitors are being expected to emerge as an important class of novel therapeutic agents in the future. Few other issues, important while performing lab experiments with natural products, are also touched upon.

Keywords Bioactive natural products • Quorum sensing • Quorum sensing inhibitors

1 Prelude

Since the start of this century, there has been an increasing interest among researchers in exploring the variety of biological activities possessed by different natural products (NPs). Though natural products (largely secondary metabolites) from both terrestrial and marine origin are being investigated, much of the work has focused on natural products of plant origin. Plant preparations have formed the core of most of the ancient systems of medicine. For example, one of the most ancient systems of medicine/healthcare—*Ayurved*—has been practiced widely in India and neighboring countries like Sri Lanka (Chopra and Doiphode 2002). *Atharvaved* (around 1200 BC), *Charaka Samhita*, and *Sushruta Samhita* (100–500 BC) are the underlying classics containing detailed descriptions of over 700 herbs (Dash and Sharma 2001). Descriptions of the use of natural substances for medicinal purposes

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can be located in texts as old as 78 A.D.; for example, *De Materia Medica*, written by Dioscorides, mentions thousands of medicinal plants (Tyler et al. 1988). In ancient times, human lifestyle was such that for every need they had to look into the nature as a source. Today, even when the mankind has developed the skill for synthesizing different molecules in the chemistry lab, we look into the nature to find new classes (i.e., novel structures) of bioactive molecules. Many of the natural secondary metabolites are large molecules with complex structures, and it is not always possible to synthesize them chemically. Few others are first extracted from some natural source, and identified as a *lead* molecule, following which that structure can serve as scaffold for synthetic products. In any case, screening natural products for the desired bioactivity remains an attractive option. In this article, we start with a short description of the bioactive natural products and then focus particularly on the natural products capable of interfering with microbial quorum sensing. Toward the end of the article, we describe some of the issues important for a natural product researcher.

NPs are the substances found in nature, i.e., synthesized by a living organism. These NPs can have one or more pharmacological or biological activities (Koehn and Carter 2005). Among these NPs, primary metabolites usually have some essential role in a cell/organism that produces them, whereas secondary metabolites generally are used by the producing organisms to perform accessory (but important) functions such as controlling natural relationships, particularly those related to defense against predation, competition for resources, interspecies communication for mating and hunting, etc. Owing to their interesting and potentially useful properties, secondary metabolites can prove to be beneficial to humans. NP can be used as therapeutic agents for managing conditions such as cancer, inflammation, bacterial infections, etc. (Bhatnagar and Kim 2010; Lv et al. 2011; Gyawali and Ibrahim 2012). Table 1 lists some of the reported therapeutic uses of certain NP. NP research holds its value as one of the most thriving sources of drugs, while offering a wide range of structural diversities and biological activities. Hitherto, only a fraction of the world's biodiversity has been investigated for biological activity, and a larger lot remains to be explored. Additionally NP research can help building the bridge between traditional wisdom and modern medicine. The active interest of international research community in NP research is evident from the search results obtained using "natural product" as a keyword. Such a search, for example, in "Google Scholar" yields more than 2.8 million results; in DOAJ, this retrieves 16 journals and more than 2,400 articles. A year-wise search performed in PubMed, using the same keyword, shows the rise in count from 2 in 1958 to >1300 in 2015. Parallel to the increase in number of participating researchers, quite a few databases (Table 2) have come into existence providing a lot of useful information relevant to natural products.

Table 1 Examples of natural products reported for various biological activities

Compound/ product	Source	Reported activity	Reference
Artemisinin	*Artemisia annua*	Antimalarial	Tu (2011)
Paclitaxel	*Taxus brevifolia*	Anticancer	Priyadarshini and Keerthi (2012)
Axisonitrile	Marine sponge *Axinella cannabina*	Antimalarial, antituberculosis, antibacterial	Perdicaris et al. (2013)
Quinine	*Cinchona pubescens*	Antimalarial	Achan et al. (2011)
Vinblastine, Vincristine	*Catharanthus roseus*	Anticancer	Sain and Sharma (2013)
Curcumin	*Curcuma longa*	Antimicrobial	Tyagi et al. (2015)
Quercetin	Found in multiple plants, e.g., *Malus domestica*, *Syzygium cumini*, etc.	Anti-biofilm	Lee et al. (2011) Kothari et al. (2011)
Pentaphyte P-5®	*Ficus benghalensis*, *Ficus religiosa*, *Ficus racemosa*, *Ficus lacor*, *Albizia lebbeck*	Anti-inflammatory, antiasthmatic, antibacterial	http://www.palepmrf.com/Pentaphyte_P5.html

Table 2 Natural product databases

Database	Content of database	Relevant weblink
Natural Health Products Ingredients Database	Medicinal and nonmedicinal ingredients	http://www.hc-sc.gc.ca
Natural Medicines Comprehensive Database	Natural product effectiveness, drug interaction, clinical information on complementary, alternative and integrative therapies	http://naturaldatabase.therapeuticresearch.com
Super Natural II	A database of natural products comprising >325,000 natural compounds, including information on the corresponding 2D structures, physicochemical properties, predicted toxicity class, and vendors	http://bioinf-applied.charite.de/supernatural_new
Natural Products Alert	Organism, pharmacology, compound, and author-based queries	https://www.napralert.org
Universal Natural Products Database	Chemical name, CAS registry number, molecular weight and formula, international chemical identifier, and molecular input line entry specification	http://pkuxxj.pku.edu.cn/UNPD/index.php
Links to the 64 Databases For Natural Products	Structures, physical characteristics, formula, author information	http://depth-first.com/articles/2011/10/12/sixty-four-free-chemistry-databases/

2 Natural Products for Dealing with Naughty Microbes

Among the variety of biological activities being looked for in the NP, one of the most common is the antimicrobial (more recently, antivirulence, too) activity. As pathogenic microorganisms have been troubling the mankind since the prehistoric times, there has been a well-practiced tradition of employing antimicrobial NP to deal with infections. Essential oils and other plant preparations have been reported to contain a large variety of bioactive secondary metabolites (Tiwari et al. 2009). Phytochemicals are being extensively studied as promising human disease-controlling agents and/or as functional food ingredients. A variety of plant metabolites with antimicrobial properties have been documented to be effective against pathogenic and spoilage microbes (Ngwoke et al. 2011). Plants as a source of natural antimicrobials have been recognized for centuries by ancient civilizations; however, over the last three decades or so, this is being increasingly confirmed using the tools of modern science (Aires et al. 2009; Gyawali and Ibrahim 2012). Animals have also evolved different antimicrobial substances/defense mechanisms over the long process of evolution. Many of the antimicrobial peptides inherent to animals help the producing host while dealing with the invasion by pathogenic microbes (Hoskin and Ramamoorthy 2008).

Animals and plants are the major hosts for the pathogenic microbes, and hence they can be naturally expected to produce a variety of antimicrobial substances as a part of their defense strategy. In addition to this, antimicrobial substances are produced by microorganisms too, for a variety of ecological purposes. In fact, most of the currently used antibiotics have come from bacteria and fungi. Metabolites such as penicillins, cephalosporins, tetracyclines, aminoglycosides, chloramphenicol, macrolides, etc. are good examples of effectively used antibiotics derived from bacteria or fungi (Demain 1999). Food industry has also exploited the ability of different microbes to produce various antimicrobial metabolites such as different organic acids, hydrogen peroxide, ethanol, diacetyl, bacteriocins, etc., for preservation and/or flavor purpose (Nes and Johnsborg 2004).

Over the very long period of their existence on earth, microbial populations had encountered in nature a wide range of naturally occurring antibiotic substances, and for becoming more fit for survival, they developed multiple resistance mechanisms (Hancock 2007).This rise of drug resistance limits the effectiveness of any of the available antimicrobials put into therapeutic use and makes it imperative for us to keep finding new antimicrobials. Another problematic dimension of this issue is the ability of pathogenic microbes to form biofilms, which can be much more (up to few hundred times) antibiotic resistant than their planktonic counterparts. Biofilm formation is one of the many traits of pathogenic microbes whose regulation is related to *quorum sensing* (QS). QS refers to the phenomenon whereby microbes communicate among themselves, within and across populations. QS-associated microbial behavior is often of high relevance from a human perspective (Hense and Schuster 2015). This is executed via small diffusible molecules and directs most members of the given microbial population to exert a common behavior.

Gram-negative bacteria employ autoinducers, viz., *N*-acyl homoserine lactones (AHLs), to coordinate gene expression in a population density-dependent fashion (Molina et al. 2003), whereas gram-positive bacteria make use of autoinducer peptides to achieve the same. When a single bacterium secretes autoinducers (AI) into the surrounding, their concentration is too less to be detected. However, when enough bacteria are present, AI concentration reaches a threshold level allowing the bacteria to sense a critical biomass and, in response, to activate or repress the target genes associated with functions like sporulation, bioluminescence, antibiotic production and resistance, biofilm formation, pathogen/host interaction, virulence factor release, etc. (Adonizio et al. 2008; Rutherford and Bassler 2012; Kalia 2014; Hense and Schuster 2015; Lixa et al. 2015).

The fact that biofilm formation and expression of several other virulence factors is linked to QS (Fig. 1) raises new hopes for the discovery and development of anti-pathogenic (i.e., antivirulence/anti-infective) drugs capable of interfering with the bacterial communication system, without necessarily inducing lethal effects (Song and Wen 2013). The anti-infective compounds are expected to exert lesser selection pressure on the target pathogens (Rasko and Sperandio 2010; Breah and Michael 2013), than that exerted by conventional antimicrobial agents acting either as microbicidal or microbiostatic agents, i.e., affecting the pathogen growth in a direct fashion. However, QS inhibitors (QSI) should not be thought as evolution-proof drugs (Allen et al. 2014). Reports of resistance to QSI have already appeared (Kalia 2013; Grandclément et al. 2015).

QSI may exert their disrupting effect by inhibiting synthesis of the signal molecule, binding with the signal molecule and thus not allowing it to reach the compatible receptor, or binding itself with the receptor and not allowing the actual signal to occupy the binding site on the signal receptor (Kalia et al. 2014; Kalia and

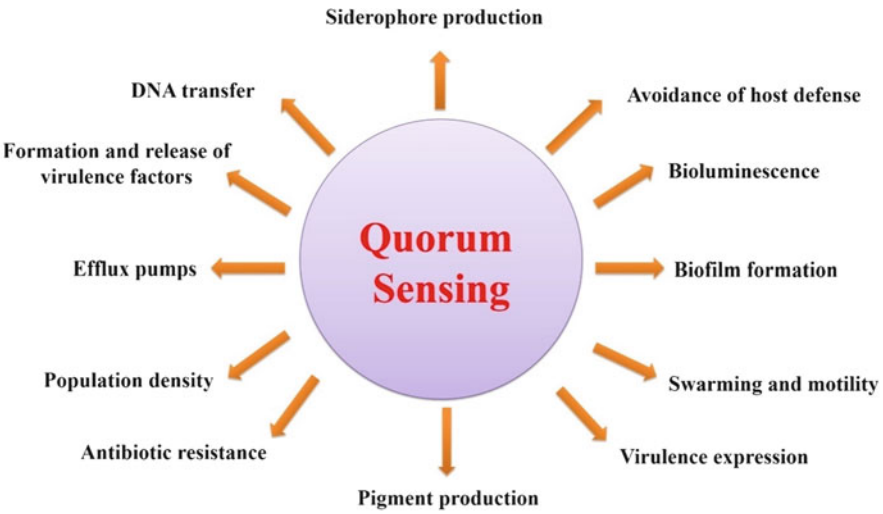

Fig. 1 A multitude of QS-regulated functions among microorganisms

Kumar 2015a). Natural as well as synthetic preparations with QS inhibitory potential are being extensively studied. Among natural entities, several plant compounds have been reported to be capable of acting as QSI (Table 3). Plants, such as carrots, chili, garlic, tomato, soybean, vanilla, pea, etc., have been shown to possess compounds having anti-QS activity (Zhu et al. 2011). Various species of marine algae, fungi, lichens, animals, honeybees, etc., are also reported to produce anti-QS compounds (Zahin et al. 2010; Lazar et al. 2013; Martín-Rodríguez et al. 2014).

As QS is put to use by multiple pathogens (e.g., *Enterococcus faecalis*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli* and those belonging to the genera *Helicobacter*, *Neisseria*, *Porphyromonas*, *Proteus*, *Salmonella*, etc.) for regulation of virulence expression (George and Muir 2007; Bhardwaj et al. 2014; Kalia 2014); the QS machinery is being viewed as a very attractive target for drug design (Kalia et al. 2014). A limited number of QSI may prove effective against a multitude of pathogens, as there are many parallels among pathogenic microbes with respect to the components/mechanisms of their QS circuit. QS inhibitory compounds are thought to emerge as a new type of antimicrobial agents with possible applications in different fields, including human and veterinary medicine, agriculture, and aquaculture. Commercial interests associated with these fields are massive, as evident from a good number of biotechnology firms, which emerged on the scene in the near past, aiming specifically at developing anti-QS formulations [for instance, QSI Pharma A/S (Denmark); Quorex Pharmaceuticals Inc., Carlsbad (USA); 4SC AG (Germany)] (Hentzer and Givskov 2003).

2.1 *Selection of the Model Bacterium for Screening of Possible QSI Property*

Researchers, while screening their test substances for possible in vitro QS inhibitory property, usually employ one or more bacteria as the model test organism, and then they investigate the effect of their test substances on one or more QS-regulated phenotypes in the selected test bacteria. Though there are quite a few QS-associated traits, pigment production is one, which can be measured relatively easily. Production of pigment in many bacteria (e.g., *Pseudomonas aeruginosa*, *Chromobacterium violaceum*, *Serratia marcescens*, *S. aureus*, etc.) is known to be associated with QS (Table 4). However, while working with colored organisms, experiments may be tricky in some way. Particularly while quantifying the bacterial growth photometrically, the experimenter must ensure that there is no interference due to light absorption by the pigment. Most pigments are likely to absorb significantly at the wavelengths commonly used (e.g., 625 or 660 nm) for measuring OD of bacterial cultures. To overcome this problem, one must prepare the absorption spectrum of the pigment produced by the test organism and should avoid quantifying microbial growth at any wavelength where pigment absorbs to any notable

Table 3 Phytochemicals reported to possess anti-quorum sensing property

Principal compound responsible for anti-QS property	Source plant	Effective against	Reference
Epigallocatechin gallate	*Camellia sinensis* L.	*S. aureus*	Blanco et al. (2005)
Gingerol	*Zingiber officinale*	*P. aeruginosa*	Kim et al. (2015)
Ellagic acid	*Terminalia chebula Retz.*	*P. aeruginosa*	Sarabhai et al. (2013)
Pyrogallol	*Punica granatum*	*Vibrio harveyi*	Sangeetha and Vijayalakshmi (2011) and Brackman et al. (2008)
Urolithin A and B	*Punica granatum*	*Yersinia enterocolitica*	Truchado et al. (2012)
Methyl eugenol	*Cuminum cyminum*	*C. violaceum*, *P. aeruginosa*, *S. marcescens*	Packiavathy et al. (2012)
Gallic acid	Found in many plants, e.g., grapes	*Salmonella typhimurium*, *Citrobacter freundii*, *Proteus mirabilis*, *S. aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *E. coli*, *P. aeruginosa*	Boussoualim et al. (2014)
Quercetin	*Guiera senegalensis*	*E. coli*	Djifaby et al. (2012)
Vanillin	*Vanilla planifolia*	*C. violaceum*	Choo et al. (2006)
Naringenin	*Citrus sinensis*	*P. aeruginosa*	Vandeputte et al. (2011)
Taxifolin	*Combretum albiflorum*	*P. aeruginosa*	Vandeputte et al. (2011)
Cinnamolide-valdiviolide	*Drimys winteri*	*C. violaceum*	Carcamo et al. (2014)
Iberin	*Armoracia rusticana*	*P. aeruginosa*	Jakobsen et al. (2012a)
Erucin	*Brassica oleracea*	*P. aeruginosa*	Ganin et al. (2013)
Ajoene	*Allium sativum*	*P. aeruginosa*	Jakobsen et al. (2012b)
Allicin	*Allium sativum*	*S. aureus*	Leng et al. (2011)
Caffeine	*Coffee arabica*	*E. coli*, *P. aeruginosa*	Norizan et al. (2013)

Table 4 QS signaling molecules and QS-associated phenotypes in some pigmented bacteria

Bacterium	QS system of the organism	Autoinducer(s)	Phenotype (s) controlled	References
P. aeruginosa	*LasI*/*LasR*	*N*-(3-Oxododecanoyl)-homoserine lactone	Biofilm formation, virulence factors expression, pyocyanin production, bioluminescence, sporulation, and mating	Zahin et al. (2010), Jimenez et al. (2012), Nazzaro et al. (2013) and Aswathanarayan and Rai (2014)
	RhlI/*RhlR*	*N*-(Butyryl)-homoserine lactone		
S. aureus	LuxS/AI-2	Autoinducing peptide (AIP1-AIP4)	Cross-signaling between strains and species, biofilm formation, virulence factor expression, staphyloxanthin production	Zhao et al. (2010) and Gordon et al. (2013)
S. marcescens	SpnIR	*N*-3-Oxohexanoyl-homoserine lactone (3-oxo-C6-HSL), *N*-hexanoyl-homoserine lactone, *N*-heptanoyl-homoserine lactone, and *N*-octanoyl-homoserine lactone	Flagellum-independent population surface migration (sliding); synthesis of biosurfactant, prodigiosin, and nuclease	Wei et al. (2006) and Lutfi et al. (2014)
C. violaceum	CviI/R	*N*-hexanoyl homoserine lactone (C6-AHL)	Violacein production, exoprotease, aggregation, biofilm formation, swarming motility	Vasavi et al. (2013) and Juarez et al. (2013)

extent. For example, *C. violaceum* is among the most widely used bacteria in QS-related experiments, and it produces the violet pigment violacein. To avoid any notable interference from violacein, bacterial growth in this case can be quantified at 764 nm (Gallardo et al. 2014), as violacein does not absorb at this wavelength. Similarly, appropriate wavelengths need to be selected while working with other pigmented bacteria.

Though screening for QSI can initially be performed using any of the suitable test bacterium, any QSI can be of some real value only when it is shown to be capable of inhibiting QS in multiple bacteria. This is to say that an ideal QSI should exert a broad spectrum of activity by being capable of interfering with the QS machinery in gram-positive as well as gram-negative bacteria. The most effective approach will be to show multiple QS-associated traits (in each of the test organisms) to get affected upon exposure to the test product.

2.2 *Possible Workflow While Mining NP for Potential QSI*

Once a test product has been demonstrated to possess a broad-spectrum in vitro capacity to inhibit quorum sensing, the next logical step can be to investigate whether this capacity can be demonstrated in vivo. For achieving the latter, availability of a suitable model host is essential. Though animal models are in use since many years, ethical issues are associated with their use. The nematode *Caenorhabditis elegans* has emerged in recent years as an attractive model host for infectious microorganisms (Ewbank and Zugasti 2011), at least for initial in vivo studies.

Following the confirmation of in vitro and in vivo activity, the next step of investigation can be to find out the mode of action of the potential QSI. For this one can take the in silico approach, if the phytochemical profile of the test plant extract is known, wherein structures of different constituent metabolites of the active extract can be docked against the possible bacterial target(s), e.g., the QS signal and/or the signal receptor protein (CviR in *C. violaceum*, as an example). Performing the in vitro experiments with and without exogenous supply of QS signal can provide useful indication on whether the potential QSI is a *signal-supply inhibitor* or a *signal-response inhibitor*. This information is of obvious utility while selecting target proteins during molecular docking exercise. Performing *wet lab* experiments with pure compounds can be of additional value. In silico exercise can run in parallel with the in vitro or in vivo experiments (Fig. 2).

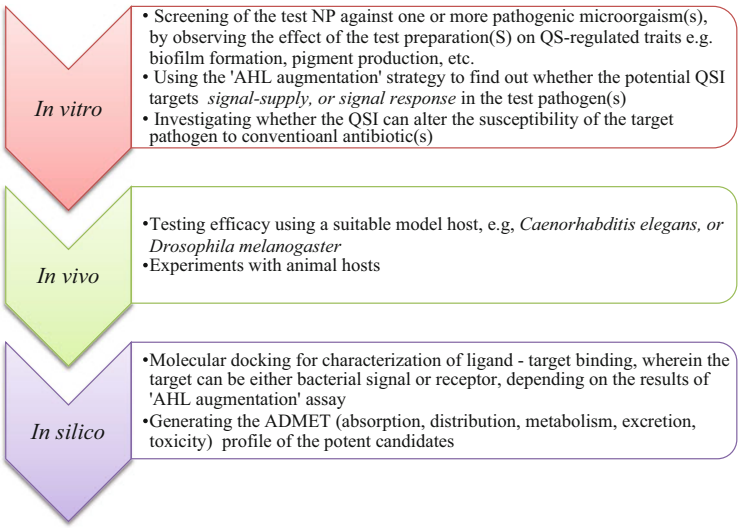

Fig. 2 An indicative list of experiments to be performed while mining NP for potential QS inhibitors

3 Applied Aspects of the QS Research

QSI seem to have varied applications in different fields including medicine (Joshi et al. 2010; Kalia and Kumar 2015b). QS research of course adds a lot to our knowledge about the fundamental aspects of microbial communication and regulation of the population behavior. Magnitude of the applied aspect of QS research is also evident from a good number of patents being filed in this area (Table 5).

3.1 QSI in Medicine

As an alternative/augmentation to the currently practiced conventional antibiotic therapy, QSI are being viewed with great hopes. In order to be therapeutically relevant, a QSI need not be 100% effective, as disturbing the QS machinery of the given pathogen even partially can reduce its virulence significantly, which in turn can offer the host immune system a better chance of winning over the pathogen. Further QSI may enhance antibiotic susceptibility of the given pathogen, making the conventional antibiotic(s) more effective at lesser concentration. QSI may act in synergy with the routinely applied antibiotics. The term "synergy" refers to the fact that the effect of combined treatment is more than the sum of each component's individual effects. Certain components in a plant extract can improve the therapeutic effect of the chemotherapeutic agents (Cooney 2011). In certain cases, one herb can enhance the effect of another, if given simultaneously (Spinella 2002). As an example, we may consider the *Panchvalkal* preparations described in *Ayurved*. These are mixtures of extracts of bark from different plants. Such preparations have been prescribed in *Ayurvedic* texts for relief in microbial infections. One such commercially available product Pentaphyte P5® is being investigated by us for its QS inhibitory potential. Our yet unpublished findings suggest that this product (listed in Table 1) can reduce QS-regulated violacein synthesis in the bacterium *C. violaceum*. It could also enhance (~10%) the susceptibility of this bacterium to the antibiotic streptomycin. It is appealing to consider the combined use of antibiotics with anti-QS strategies, since QSI by disrupting bacterial signal production/reception can reduce antimicrobial resistance (e.g., by reducing drug efflux) or discourage transition to physiological states that enhance persistence (e.g., biofilms). Many such reports describing the benefit of using QSI in combination with antibiotics have accumulated in literature (Rasmussen et al. 2005; Brackman et al. 2009, 2011), which show the combination approach to be more effective against pathogens like *P. aeruginosa*, *Burkholderia* spp., *S. aureus*, etc. QSI compounds were also shown to improve survival probabilities in invertebrate infection models and to decrease bacterial load in mouse pulmonary tissues (Brackman et al. 2009). A rise in the antibiotic susceptibility of the test bacterial strains was attributed to the synergistic activity of quercetin (Venkadesaperumal

Table 5 Some examples of the patents related to QS research[a]

Sr. no.	Patent title	Inventor(s)	Reference no. and date of publication
1	Development of zinc oxide nanoparticles at varied incubation periods for regulating anti-quorum sensing	Khan Mohd Farhan, Ansari Akhter H	IN2232DE2015 (A),2015-08-14
2	Small-molecule antagonists of bacterial quorum sensing receptors	Bassler Bonnie L, Swem Lee R	US2015306067 (A1), 2015-10-29
3	Bacterial quorum sensing inhibitor and antibacterial application thereof	Yu Wengong, Gong Qianhong	CN104784160 (A), 2015-07-22
4	Modulation of bacterial quorum sensing with synthetic ligands	Blackwell Helen E, Geske Grant D	US2015080349 (A1), 2015-03-19
5	Antibody-mediated disruption of quorum sensing in bacteria	Kim D Janda, Gunnar F Kaufmann	JP2014221774 (A), 2014-11-27
6	Quorum sensing inhibitors	Givskov Michael, Yang Liang	WO2014142748 (A1), 2014-09-18
7	Use of ellagitannins as inhibitors of bacterial quorum sensing	Mathee Kalai, Adonizio Allison L	US2013317094 (A1), 2013-11-28
8	Detecting antigens such as bacterial quorum sensing proteins	Bell Charleson S, Giorgio Todd D	WO2013170229 (A1), 2013-11-14
9	Bacterial quorum sensing biosensor	Sayre Richard T, Rajamani Sathish	US2012122115 (A1), 2012-05-17
10	Synthetic analogs of bacterial quorum sensors	Iyer Rashi, Ganguly Kumkum	US2012071430 (A1),2012-03-22;US8350061 (B2) 2013-01-08

[a]This table was generated by performing a search using the keyword "quorum sensing" on the website of European Patent Office: https://www.epo.org/searching.html. This search yielded >340 results, of which few examples are listed here

et al. 2015). Such investigations may pave the way for novel treatment options for dealing with "difficult-to-eradicate" bacterial infections.

3.2 QSI in Aquaculture and Agriculture

In commercial aquaculture, bacterial infections are one of the most critical problems. Vibriosis is known to cause heavy mortality in almost all types of aquacultured organisms (Defoirdt et al. 2007). Natural and synthetic brominated furanones were shown to protect brine shrimps (*Artemia franciscana*) from pathogenic isolates of *Vibrio* (*V. harveyi*, *V. campbellii*, and *V. parahaemolyticus*) through the disruption of AI-2-based QS (Defoirdt et al. 2006).

In agriculture, nonpathogenic bacteria capable of disrupting QS of the phytopathogenic bacteria can be used as biocontrol agents (Dong et al. 2004; Uroz et al. 2008). QS-regulated virulence in plant pathogens, including the soft rot associated with *Pectobacterium* spp., was shown to be disrupted by some QSI (Faure and Dessaux 2007).

3.3 QSI as Anti-biofouling Agents

Biofouling can be defined as the attachment of one or more organisms to a surface in contact with water. This phenomenon causes serious technological and economic problems in various fields or processes such as naval transportation, aquaculture, petroleum industries, medical devices, bioreactors or water distribution networks, and wastewater plants (Fitridge et al. 2012; Harding and Reynolds 2014). Marine organisms constitute a good source of antifouling molecules. *Flustra foliacea*, a marine colonial animal of the Bryozoa phylum, produces a set of ten brominated alkaloids, two of which exhibit QSI activity (Peters et al. 2003). In glass plate assays, kojic acid, an oxo-pyrone, prevented biofouling (Dobretsov et al. 2011). *Piper betle* extracts were indicated as anti-QS agent to mitigate membrane biofouling (Siddiqui et al. 2012).

4 Issues While Experimenting with NP

Natural products, particularly crude extracts, being undefined preparations pose certain challenging issues, while investigating them for different biologically relevant activities. Some of the important aspects of natural product research, which researchers should be conscious about, include:

- Batch-to-batch variation
- Selection of the most appropriate extraction method
- Appropriate "controls" in all experimental sets (particularly the "abiotic control" while dealing with colored extracts in a study involving photometric measurements) (Chaudhary et al. 2014; Wadhwani et al. 2009)
- Low solubility in the assay medium
- Existence of the phenomenon of "synergy," making it difficult to get a clue about mode of action
- Lack of globally accepted authentic guidelines regarding protocols for assaying NP and their therapeutic uses

Few suggestions for troubleshooting with NP issues can be found in Kothari (2014).

5 Conclusions

Research on bioactive natural products is being intensively practiced across the globe. NPs with antimicrobial and/or anti-infective potential are getting more and more attention in the background of the threatening problem of antibiotic resistance among pathogenic microbes. Particularly the NP with QS inhibitory potential are being viewed with high optimism, as QS regulates a notable portion of the microbial genome, including that associated with their virulence. QSI are expected not to persuade bacteria toward rapid development of resistance. They may be used alone or in combination with conventional microbiostatic/microbicidal agents. It is believed that QSI can help the host immune system by reducing the expression of virulence traits, as well as potentiate the effect of antibiotic therapy by making the target pathogen population more susceptible. Though many reports on QSI potential of NP are appearing, the real challenge will be to develop these active NP as usable therapeutic agents. We also need to develop some insight into how the normal human microbiota may respond to the QS inhibitory natural products, if employed as therapeutic agents. NP research is an interesting area, but having its own complications. However, there are enough reasons to believe that the future will see a good number of NPs entering the list of approved therapeutic formulations. A structured approach of research in this area will help us to explain the scientific basis of many of the traditional medicinal practices, for example, the use of pomegranate peel for relief in sore throat, or applying coffee powder on wounds. Natural product researchers can play a crucial role in bridging the gap between ancient and modern systems of medicine.

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Fungi Imperfecti Laccase: Biotechnological Potential and Perspectives

Bhagwan Rekadwad and Chandrahasya Khobragade

Abstract Laccase is a multicopper oxidase enzyme (EC 1.10.3.2, benzenediol: oxygen oxidoreductase) which belonged to polyphenol oxidases. A laccase enzyme is produced by plant, bacteria, insects, and fungi. Among the fungi, laccase is produced by many sexually reproducing fungal species and occupied their position in established taxonomic classifications of fungi. In contrast, laccase is also produced by other asexually reproducing fungi. These are called as Fungi imperfecti/Deuteromycetes. Laccases produced by deuteromyces are used in various biotechnological applications such as wastewater treatment, detoxification or discoloration of industrial effluents, dye degradation, bleaching of pulp and papers and textiles in industries, biofuel or bioethanol production, wine and beer making, synthesis of organic compounds, and bioremediation—for degradation of pesticides, polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB), synthetic polymers, explosives, and synthetic dyes. It will definitely have great contribution in diverse settings.

Keywords Agriculture • Biomedicine • Box-Behnken methodology • Chitinase • NAG • Taguchi method

1 Introduction

Multicopper oxidase enzyme containing copper (Cu) atoms in the catalytic center is called as laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductase). Laccases belonged to the broad group of enzymes called polyphenol oxidases. There are three kinds of laccases, blue laccase containing three atoms and yellow or white laccase, both lacking blue Cu atoms. This difference in laccase can be distinguished using ultraviolet/visible light and EPR spectra. In blue copper, blue color to the protein (laccase) is imparted by T1 copper at an absorbance 600 nm with a detectable EPR. In yellow laccase, there is no color conferred by T2 copper but is

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EPR detectable. For white laccase, yellow color to the protein (laccase) is imparted by a pair (two) of T3 copper atom in the near UV range and no EPR signal is detectable. Different active sites are described for laccases in the literature by various research groups worldwide. Typically laccases are acting as a mediator and catalyze a reaction in which water molecule is formed with the reduction of oxygen and oxidation of the substrate. Laccase has oxidized ranges of chemicals such as polyphenols, aromatic diamines, methoxy-substituted phenols, etc. (Pozdnyakova et al. 2006; Baldrian 2006; Morozova et al. 2007; Valls et al. 2010).

Laccases are produced by a wide range of organisms including plants, bacteria, insect, and fungi (Kunamneni et al. 2007). A laccase enzyme is produced by plant, bacteria, insects, and fungi. Among the fungi, laccases are produced by many sexually reproducing fungal species and occupied their position in established taxonomic classifications of fungi. In contrast, laccase is also produced by other asexually reproducing fungi. These are called as Fungi imperfecti/Deuteromycetes. Fungi imperfecti are also known as Deuteromycetes (Greek name "second fungi"), which reproduces only asexually through sporogenesis and with no sexual spores. These fungi are also termed as mitosporic fungi or anamorphic fungi without any taxonomic rank. *Ascomycota* (*Ascomycetes*), *Basidiomycota* (*Basidiomycetes*), and *Deuteromycota* (*Deuteromycetes*) are the most frequent terms used to denote Fungi imperfecti. Among these, the lignin-degrading white-rot fungi were particularly most abundant.

Laccases produced by the *Ascomycetes*, *Basidiomycetes*, and *Deuteromycetes* are playing a very important role in many biotechnological applications. Because of their wide temperature and pH range, they have a large capacity to utilize phenolic and non-phenolic lignin and related compounds as well as xenobiotic chemicals. These key features of laccases have profound biotechnological applications such as in wastewater treatment, detoxification or discoloration of industrial effluents, dye degradation, bleaching of pulp and papers and textiles in industries, biofuel or bioethanol production, wine and beer making, synthesis of organic compounds, and bioremediation—for degradation of pesticides, polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB), synthetic polymers, explosives, and synthetic dyes (Desai and Nityanand 2011; Brijwani et al. 2010; Zhao et al. 2012; Vadapally et al. 2015).

2 Recombinant DNA Technology: Preparation, Cloning, and Expression of Synthetic Genes Encoding Laccase

The recombinant DNA technology is a useful tool to clone laccase-encoding gene to the other microorganisms capable of withstanding in harsh conditions. Fungi *Ganoderma lucidum* and *Pleurotus ostreatus* are the producers of extracellular laccase. Laccase-encoding genes GlLCC1 in *Ganoderma lucidum* and POXA 1B in *P. ostreatus* were evaluated. These were optimized for codon use, GC percentage, and secondary structures. Computational model was constructed to evaluate

the interaction of laccase with substrate. This means molecular docking acts as limelight for evaluation of laccase interaction with 2,20-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS). Artificially synthesized new (synthetic) genes were cloned. This process was controlled by *Pichia pastoris* glyceraldehyde-3-phosphate (GAP) dehydrogenase constitutive promoter. The transformed recombinant *P. pastoris* was generated using pGAPZαA-LaccGluc-Stop and pGAPZαA-LaccPost-Stop constructs. After optimization, the GC content of LaccGluc-Stop and LaccPost-Stop genes was reduced by 47% and 49%, respectively. Both genes have 0.84 codon adaptation index. 3D structure analysis was performed using SuperPose software. It is revealed that LaccGluc-Stop and laccase crystallographic structure 1GYC of *Trametes versicolor* mimic each other. These produced results were validated through 3D model/ABTS (Baker and Sali 2001; Piontek et al. 2002; Wang et al. 2009; Poutou-Piñales et al. 2010; Sun et al. 2012; Huifang et al. 2013; Rivera-Hoyos et al. 2013). Hence, the optimization of gene GlLCC1 (isolated from *G. lucidum*) and gene POXA 1B (isolated from *P. ostreatus*) were expressed in *P. pastoris* under control of GAP promoter and α-factor leader. The recombinant *P. pastoris* containing cloned genes (GlLCC1 and POXA 1B) is an ideal design for construction of environmentally friendly recombinant *Deuteromycetes* expression systems. It will definitely have great contribution in diverse settings: chemical use, clinical, environmental and industrial applications (Bertrand et al. 2002; Dundas et al. 2006; Wang et al. 2006; Morris et al. 2009; Santo et al. 2013; Rivera-Hoyos et al. 2015).

3 Biotechnological Applications of Microbial Laccase

3.1 In Synthesis of Organic Compounds

Blue laccase (blue multicopper oxidase) carries out oxidation reactions. It is suitable for organic synthesis of phenolic and nonphenolic compounds and environmental pollution treatment. It converts organic compound into nontoxic or less toxic form through its oxidation, polymerization, dimerization, and amination (Mogharabi and Faramarzi 2014).

3.2 Biodecoloration and Biodegradation of Textile Dyes

Textile industries consume in total two-thirds of different dyestuff, requiring a huge quantity of water and lot of inorganic polymer compounds and organic chemicals for wet processing. This uncontrolled use results in the generation of bulk colored wastewater which may be composed of synthetic dyes, which have resistance to biodegradation. Nowadays, cleaning and decolorization of generated wastewater before discharge is a compulsory practice as per environmental regulations in most

of the countries. Wastewater from textile industries contains a high amount of colored dyes that are toxic in nature, increased salinity, and pH of wastewater. Dumping of these colored dyes into the water bodies seriously affects the aquatic life. White-rot fungi produce laccases, which are useful for oxidation of toxic and colored textile wastewater, resulting in the production of insoluble products. Subsequent separation of produced precipitate is the most promising and fast approach for decoloration and purification of textile wastewater (Wesenberg et al. 2003; Forgacsa et al. 2004; Travares et al. 2009). Commercially, these multicopper oxidases were used for improving the whiteness of cotton (bio-bleaching), bio-stoning of gens, denim processing, conversion of dye precursor for most efficient fabric dyeing, and cloth and dish washing and, in detergents, as an anti-shrink agent for treatment of wool fibers (Raghukumar 2000; Mougin et al. 2002; Lantto et al. 2004; Kiro 2013). The microorganisms capable of withstanding high salinity and pHs are needed to decolorize and detoxify the textile dyes (Devi et al. 2012). A *Deuteromycetes* fungus, *Pestalotiopsis* sp., NG007, has a strong ability to decolorize Reactive Red 4 dye under the saline condition and at pH 8.0. The immobilized culture of *Pestalotiopsis* is also able to decolorize three textile dyes such as Reactive Red 4, Reactive Green 19, and Reactive Orange 64 with very high efficiency (20–98%) of discoloration at a varied range of pH 3.0–12 and at a salinity 0–10% w/v over a period of 3 days (Yanto and Tachibana 2013; Yanto et al. 2014). Another *Ascomycetes* fungus, *Paraconiothyrium variabile*, is capable of decolorization of five (5) synthetic dyes. It has decolorized 93% of Remazol Brilliant Blue R (RBBR) (at 600 mg/L initial concentration) after 3 h of treatment in the presence of 5 mM hydroxybenzotriazole (Aghaie-Khouzani et al. 2012). Laccase-producing marine *Basidiomycota* fungi decolorized 50–99% of 0.01% Remazol Brilliant Blue R dye. These marine *Basidiomycota* have produced 528 U/L and 643 U/L laccase and decolorized the RBBR (Azri et al. 2015). Similarly, a novel *Deuteromycete*s fungus *Myrothecium verrucaria* NF-05 was isolated from soil collected from Liangshui Nature Reserve located in China. Soil samples were studied using central composite design and response surface analysis. It is revealed that the optimum concentrations of glucose, $CuSO_4$, and gallic acid for *M. verrucaria* NF-05 are found to be 26.47 g/L, 236.3 μM, and 138.4 μM, respectively. This optimization of *M. verrucaria* NF-05 production parameters leads to 1.66-fold increase in laccase production, i.e., 12,000 to 19,940 U/L laccase production is noticed (Zhao et al. 2015). Reactive Blue 49 decoloration activity of crude laccase produced by *Trametes versicolor* strain 2008001 carried out using Taguchi and Box-Behnken methodologies. It is reported that 98% decolorization of Reactive Blue 49 could be obtained under the optimum conditions (at pH 2.95, 55.6 mg/L initial dye concentration, 0.76 ml laccase/L, and reaction time 46.91 min) (Gedikli et al. 2014).

3.3 Bioethanol Production

Bioethanol is an essential biofuel obtained from the fermentation of lignocellulose. Lignocellulose is a combination of lignin, cellulose, and hemicelluloses. Production

of bioethanol from lignocellulose involves three major steps such as pretreatment of lignocellulose materials, saccharification, and fermentation (Nigam et al. 2009; Dias et al. 2010; Salvachua et al. 2011). Most of the lignin degraders belonged to white-rot fungi (Wan and Li 2011). The enzymatic system is possessed by white-rot fungi such as *Ceriporiopsis subvermispora*. The enzymatic system encompasses three principal enzymes such as laccase, lignin peroxidase, and manganese peroxidase. These enzymes have effectively carried out delignification of paper, animal feed, and biofuel production materials. Ligninolytic enzymes produce biofuel/bioethanol from lignin through delignification (Li et al. 2008; Lu et al. 2010) and detoxification (toxic compound—furan derivatives, pentose, hexose, weak acids) and degradation of phenolic compound (Palmqvist and Hahn-Hägerdal 2000; Martín et al. 2002; Chandel et al. 2007; Minussi et al. 2007; Kolb et al. 2012; Plácido and Capareda 2015).

3.4 Wine and Beer Making

Ligninolytic enzymes like laccase have main application in wine and beer stabilization. Wine and beer prepared from cheap agricultural products such as grapes, orange, molasses, and other fruits/berries have huge amount of polyphenols. The polyphenols are the contributors for the formation of undesired color/discoloration, haze, and imparts unwanted taste (flavor) to wine and beer. Polyphenols and other compounds change the organoleptic characteristics of wine and beer. Thus, complete removal of residual stem threads from the wine is essential. The use of laccase in addition and in combination with complexing agents, enzyme inhibitors, and sulfate compounds better removes phenolics and is an attractive alternative (Morozova et al. 2007; Borras et al. 2008; Rameshaiah and Reddy 2015).

3.5 Degradation of Xenobiotics

Laccases are able to oxidize a broad range of substrate and exhibit specificity toward pesticides, chlorinated phenolics, and polycyclic aromatic hydrocarbons (arise from both artificially synthesized and fossil fuels). *Coriolopsis gallica* has produced a laccase capable of oxidizing carbazole, fluorine, dibenzothiophene, and *N*-ethylcarbazole in the presence of mediators—1-hydroxybenzotriazole and 2.2′-azinobis-(3-ethyl-benz-thiazoline)-6-sulfonic acid (Bressler et al. 2000; Pointing 2001; Torres et al. 2003; Pozdnyakova et al. 2004). The phenolic compound and aromatic amines include free radicals and quinines were removed from using laccase through polymerization and precipitation (Niku-Paavola and Viikari 2000). Laccases produced by *Trametes hirsuta*, white-rot fungus, degrade alkenes by a two-step process. Firstly, laccase enzyme catalyzed oxidation ($oxid^n$) of the primary substrate. Secondly, the added mediator carried out oxidation of the secondary

substrate (alkene) to its respective aldehyde/ketone. In soil, laccase performs a process that mimics humic acid synthesis. This results in immobilization of soil pollutants (Baldrian and Gabriel 2002; Lo et al. 2002; Shraddha et al. 2011; Pannu and Kapoor 2014). These soil pollutants (xenobiotics) were immobilized by microbial laccase in such a way that their bioavailability was reduced. This in turn reduces their toxic effects on other flora and fauna. The laccase-producing yeast, *Pichia pastoris*, was genetically modified by site-directed mutagenesis. The produced genetically engineered laccase improves the electron transfer between the copper-containing active site and the electrode. This approach is useful, particularly, in the bioremediation of xenobiotic compound and recalcitrant compounds (Stack et al. 2005; Steinle et al. 2010; Viswanath et al. 2014; Capone et al. 2015).

3.6 Micropollutant Degradation

Pharmaceutical micropollutants (nonsteroidal anti-inflammatory drugs—NSAID), namely, diclofenac (DFC), meclofenamic acid (MFA), ibuprofen (IBF), ketoprofen (KFN), and naproxen (NPX), are present in wastewater in relatively high concentrations (Li et al. 2007; Samaras et al. 2010). *Deuteromycetes*, *T. versicolor*, laccase capable of degrading NSAID. *Trametes* laccase has 20 times greater activity as compared to bacterial laccases produced by *Streptomyces* (*S. cyaneus*, *S. ipomoea*, *S. griseus*, *and S. psammoticus*). *Trametes* laccase preparations perform oxid^{n} of the micropollutants. These are proved to be one of the best candidates for removal of micropollutants in municipal wastewater treatment plant (WWTP) effluents (Margot et al. 2013).

4 Future Perspectives

Laccase-producing *Deuteromycetes* may be useful as the whole microorganism, in consortia and purified laccase in environmental settings (Fig. 1).

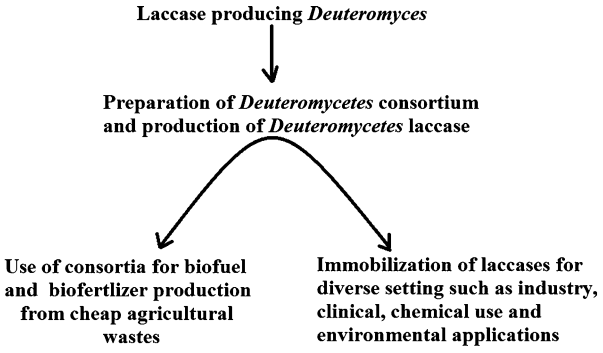

Fig. 1 Overview of different applications of *Deuteromycetes* and their laccases

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Biosurfactants: A Multifunctional Microbial Metabolite

Neha Panjiar, Shashwati Ghosh Sachan, and Ashish Sachan

Abstract During the 1960s, the science of biosurfactants was in its infancy and received attention mainly as hydrocarbon dissolving agents. Recently biosurfactants have emerged as the most versatile product of the modern microbial biotechnology. Owing to its biodegradable nature, it has been considered as "green chemical." Recently, increasing awareness toward sustainable ecosystem and environmental protection has resulted in a concerted research in biosurfactant as a promising substitute of chemical surfactants. It can be produced from nonrenewable resources, with alternative synthesis from economical renewable feedstocks. Apart from the stability at relatively adverse environments, these compounds are readily biodegradable in the environment. Functional properties of these compounds are being conferred by their structural diversity which varies among the microbial community. These compounds may provide advantages in a particular ecological niche or may be responsible for the niche-specific behavior of the producer microorganism. Diverse properties encompassed by biosurfactants are wetting, dispersion, emulsification, foaming, cleansing, phase separation, reduction in viscosity, and surface activity. It serves a broad spectrum of industries like petroleum, paint, paper, textile, leather, agriculture, cosmetic, food processing, agriculture, and pharmaceutical industries, thereby plays a significant role in white biotechnology.

Keywords Biosurfactants • Bioemulsifiers • Amphiphilic • Classification • Screening

1 Biosurfactants: A Sustainable Substitute to Conventional Surfactants

A global shift toward the use of renewable and biodegradable compounds at an industrial level has resulted in an increased interest toward natural surfactants and emulsifiers of microbial origin (Panjiar et al. 2015). Biosurfactants (surfactants of

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microbial origin) or "green surfactants" refers to the large group of structurally diverse amphiphilic compounds containing both hydrophilic (head) and hydrophobic (tail) moieties (Abdel-Mawgoud et al. 2010a; Vijayakumar and Saravanan 2015). The tail generally consists of a hydrocarbon chain of different lengths, whereas the head may be composed of carbohydrate, peptides or proteins, phosphate, hydroxyl, or an ester group (Pacwa-Płociniczak et al. 2011). Due to its amphiphilic nature, it tends to allocate itself at the air/water or oil/water interface and lowers the surface and interfacial tension, respectively. Diversity of the microbial community is responsible for the varied chemical nature of the biosurfactants produced. Biosurfactants can be broadly classified as low-molecular-mass compounds and high-molecular-mass polymers (Ron and Rosenberg 2001). Reduction of surface and interfacial tension is the main activity of the low-molecular-mass biosurfactants, whereas high-molecular-mass biosurfactants, apart from being surface active, have emerged as efficient emulsion-stabilizing compounds and are called bioemulsifiers (Lovaglio et al. 2015). Therefore, biosurfactants form a broad category of surface-active agents which include bioemulsifiers having emulsion-stabilizing ability, thus producing stable water-in-oil or oil-in-water emulsions.

A survey on world surfactants' market indicates that the global demand will reach a market value of US $36,518 million by 2017, increasing at a compound annual growth rate of 6.19% (http://www.presskontakt.se/pressreleaser/visa/pressrelease/555885/globalsurfactant/280D60AE-2833-1833-1834-AE5F70DA7152). Another analysis named "Biosurfactants Market—Global Scenario, Raw Material and Consumption Trends, Industry Analysis, Size, Share and Forecasts, 2011–2018" (http://www.transparencymarketresearch.com/), published by Transparency Market Research, indicated that that global biosurfactant market was worth US $1735.5 million in 2011 and is expected to reach US $2210.5 million in 2018. Its enormous consumer demand is currently met by synthetic counterparts, which are mainly petroleum based. Synthetic surfactants and their by-products are environmentally hazardous and toxic, as they are nonbiodegradable which may bioaccumulate in the food chain.

Biosurfactants seem to be a promising and sustainable substitute for chemical surfactants, encompassing advantages like biodegradability, biocompatibility, low toxicity, high specificity, chemical and functional diversity, rapid yet controlled inactivation, ability to be produced from renewable and largely available economical raw materials, relative ease of preparation, and functionality under adverse conditions (Lovaglio et al. 2015). Additional advantages include stability at relatively high temperature as well as in extreme environments, specificity, and readily biodegradable nature in the environment. It can be produced from nonrenewable resources as well as from economical renewable feedstock. Structural and functional diversity, biodegradable nature, and lower toxicity of these compounds, as compared to synthetic surfactants, led to an increased momentum for further research to meet the ever-increasing consumer demand for these compounds (Shete et al. 2006; Perfumo et al. 2009).

2 Classification of Biosurfactants

The classification of biosurfactants is mainly done according to their chemical structures and microbial origin (Marchant and Banat 2012). Their hydrophilic moiety consists of amino acids or cyclic peptides; mono-, di-, or polysaccharides; or phosphates whereas hydrophobic moiety comprises of unsaturated or saturated long-chain fatty acids or their derivatives (Banat et al. 2000). Ron and Rosenberg (2001) have suggested one more method to broadly classify the microbial surfactants into low-molecular-mass molecules and high-molecular-mass polymers. Low-molecular-mass compounds can lower surface and interfacial tensions as compared to high-molecular-mass polymers, which are efficient emulsion-stabilizing compounds. Low-molecular-mass surfactants include glycolipids, lipopeptides, and phospholipids, whereas high-molecular-mass polymers comprise of polymeric and particulate surfactants. Several reviews have discussed the different classes of biosurfactants and their properties (Gautam and Tyagi 2006; Pacwa-Płociniczak et al. 2011). Each class with its representative biosurfactants and microbial origin is compiled in Table 1.

3 Microbial Biosurfactant Productions: Physiological Role Played and Their Biosynthesis

Microorganisms have emerged as a ubiquitous producer of diverse compounds exhibiting surface activity similar to synthetic surfactants. Produced biosurfactant/bioemulsifier is either secreted extracellularly or remains adhered to the cell's surface. Several research focusing and describing production of biosurfactants by bacteria, yeasts, and fungi have been carried out over the past few years. Reports are available for the production of surface-active secondary metabolites by different microorganisms like *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Rhodococcus*, *Halomonas*, *Enterobacter*, *Arthrobacter*, and yeast (Maneerat 2005; Das et al. 2008). Major classes of biosurfactants with their microbial origin, nature of sample, and source are listed in Table 2. Recent research have resulted in registration and grant of several patents on different biosurfactants and bioemulsifier, their production method, applicability in various industries, and the producer microorganisms (Shete et al. 2006).

Biosurfactants/bioemulsifiers being produced by diverse microorganisms have varied structural and chemical characteristics. Functional properties of these metabolites are being conferred by their structural diversity. Variation in their chemical nature has been observed among the microbial community. Generalization about the natural role played by biosurfactants becomes difficult due to their structural and functional diversity. These compounds may provide advantages in a particular ecological niche or may be responsible for the niche-specific behavior of the producer microorganism (Ron and Rosenberg 2001). Usually biosurfactants have

Table 1 Biosurfactant classification and their microbial origin

Class of biosurfactant		Microbial origin	References
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa*	Jadhav et al. (2011)
	Trehalolipids	*Rhodococcus erythropolis*	Muthusamy et al. (2008)
	Sophorolipids	*Candida batistae*	Konishi et al. (2008)
		Trichosporon asahii	Chandran and Das (2010)
	Mannosylerythritol	*Candida antarctica*	Felse et al. (2007)
Lipopeptides and Lipoproteins	Surfactin	*Bacillus subtilis*	Kim et al. (2009)
	Iturin	*B. subtilis*	Yuan et al. (2012)
	Fengycin	*B. subtilis*	Wei et al. (2010)
	Lichenysin	*B. licheniformis*, *B. subtilis*	McInerney et al. (1990), Nerurkar (2010)
	Serrawettin	*Serratia marcescens*	Lai et al. (2009)
	Viscosin	*P. fluorescens*	Banat et al. (2010)
Phospholipids		*Acinetobacter* sp.	Kosaric (2001)
Surface active antibiotics	Daptomycin	*Streptomyces roseosporus*	Miro et al. (2012)
	Entolysin	*Pseudomonas* sp.	Reder-Christ et al. (2012)
	Echinocandin	*Papularia sphaerosperma*	Cortes and Russi (2011)
Fatty acids/ Neutral lipids	Bile acids	*Myroides* sp. SM1	Maneerat (2005)
	Polyol lipids	*Rhodotorula glutinis*	Amaral et al. (2006)
Polymeric biosurfactants	Emulsan	*Acinetobacter calcoaceticus* RAG-1	Ron and Rosenberg (2001)
	Alasan	*A. radioresistens*	Toren et al. (2001)
Particulate biosurfactants	Vesicles	*A. calcoaceticus*	Kappeli and Finnerty (1979), Chakrabarti (2012)
	Whole microbial cells	*Cyanobacteria*	Barkay et al. (1999)
		Alcanivorax borkumensis	Sevilla et al. (2015)

both hydrophobic and hydrophilic parts resulting into a tendency to distribute at the interface of air/water or water/oil, thereby causing considerable changes in surface activity, viscosity, and wettability. They may act as emulsifiers, wetting agents, dispersants, detergents, and foaming agents. Various roles played by these compounds in microbial physiology have been hypothesized by Ron and Rosenberg (2001). They enhance the availability of hydrophobic substrates by increasing their water solubility, thereby rendering them more prone to microbial degradation; reduce heavy metal toxicity by binding them; regulate the process of microbial adherence to the particular surface, associated with microbial virulence; and possess antimicrobial activity (Banat et al. 2000).

Hydrocarbon and carbohydrate are the two primary metabolic pathways that are involved in the synthesis of the hydrophobic and hydrophilic moieties of the biosurfactant, respectively, by utilizing specific sets of enzymes. Different

Table 2 Different sites and samples for isolation of biosurfactant-producing microorganisms

Nature of sample	Source	Type of biosurfactant produced	Microbes isolated	References
Undisturbed and contaminated arid southwestern soil samples	NA	NA	*Flavobacterium* sp.	Bodour et al. (2003)
Hydrocarbon contaminated industrial wastewater samples	NA	Rhamnolipids	*Renibacterium salmoninarum* 27BN	Christova et al. (2004)
Brackish water sample, river Vélez, Torre del Mar	Málaga, Spain	Lipopeptides related to the surfactin	*Bacillus velezensis*	García et al. (2005)
Crude oil and injection water samples from Iranian oil wells	Iran	NA	Mesophile and thermophile *Bacillus* sp.	Sepahy et al. (2005)
Seawater sample (temperature is 5.8 °C) from nonpolluted Norwegian fjord	Norway	NA; capable of degrading hydrocarbons	Members of the phyla *Proteobacteria* or *Bacteroidetes*	Brakstad and Bonaunet (2006)
Sediments from coastal region	Goa, India	NA	*Streptomyces* sp. S1	Kokare et al. (2007)
Marine water samples	NA	Glycoprotein	*Halomonas* species TG39 and TG67	Gutiérrez et al. (2007)
Soil sample from Antarctica	Antarctica	Glycolipid biosurfactant	*Pantoea* sp. strain A-13	Vasileva-Tonkova and Gesheva (2007)
Soil sample from diesel-run electricity-generating plant of Adekunle Ajasin University	Akungba-Akoko, Nigeria	NA	*Bacillus*, *Pseudomonas*, *Arthrobacter*, *Proteus*, *Corynebacterium*, *Micrococcus*, and *Klebsiella*	Balogun and Fagade (2008)
Soil sample from an oil field, Yellow River Delta	China	NA	Three bacterial strains L1, L2, L3	Cheng et al. (2008)
Soil sample from aircraft tank (AT) and from unused fuel in the Joint Hydrant Storage Tank (JHST)	Nigeria	NA, able to degrade International Jet A-1 Aviation Fuel	*B. subtilis*, *B. megaterium*, *Flavobacterium odoratum*, *Sarcina flava*, *Micrococcus varians*, *P. aeruginosa*, *B. licheniformis*,	Itah et al. (2009)

(continued)

Table 2 (continued)

Nature of sample	Source	Type of biosurfactant produced	Microbes isolated	References
			B. cereus, *B. brevis*, *C. tropicalis*, *C. albicans*, *Saccharomyces estuari*, *S. cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *Cladosporium resinae*, *Penicillium citrinum*, and *P. frequentans*	
Marine water sample from Tuticorin harbor	India	Lipopeptide	*Azotobacter chroococcum*	Thavasi et al. (2009)
Sediment from oil-contaminated mangrove	Guanabara Bay, Rio de Janeiro, Brazil	NA	*Microbacterium* sp.	Aniszewski et al. (2010)
Campus do Pici Wastewater Treatment Station	Federal University of Ceará, Brazil	NA	*Bacillus subtilis* LAMI008	Barreto et al. (2010)
Petroleum-contaminated soil sample	Zhongyuan Oilfield, China	Exopolysaccharide (EPS)	*Enterobacter cloacae* variant (MU-1)	Hua et al. (2010)
Healthy human skin	Pune	Proteoglycan	*Acinetobacter* sp.	Jagtap et al. (2010)
Freshwater sample from Arctic Archipelago	Svalbard, Norway	Pseudofactin I and pseudofactin II (lipopeptides)	*P. fluorescens* BD5	Janek et al. (2010)
Soil sample from Korea University paddy field	Seoul, South Korea	EPS	*Acinetobacter* sp. strain DR1	Kang and Park (2010)
Marine sponge (*Dendrilla nigra*)	Southwest coast, India	Glycolipid	*Brevibacterium aureum* MSA13	Kiran et al. (2010a)
			Nocardiopsis lucentensis MSA04	Kiran et al. (2010b)

Sea mud from the oil-polluted area	Donghai, China	NA	*Alcanivorax* sp.	Liu et al. (2010)
Soil contaminated with Fuel Oil No. 6 and used mobile oil	Mexico	NA	*Pseudomonas*, *Rhodococcus*, *Bacillus*, *Acinetobacter*	Ramos et al. (2010)
Spoiled apple	NA	Mono- and di-rhamnolipid congeners: R1C10C10, R1C10C12:1, R1C10C12, R2C10C10, R2C10C12:1, and R2C10C12	*P. aeruginosa* MA01	Abbasi et al. (2012)
Formation water sample	Jharia coalbed, India	Rhamnolipid	*Pseudomonas stutzeri*	Singh and Tripathi (2013)
Petroleum-contaminated soil sample	Port of Recife, Pernambuco, Brazil	NA	*Geobacillus stearothermophilus* UCP 986	Jara et al. (2013)
Brine water sample	Kovalam solar salt works, Kanyakumari, India	Lipopeptide	*Kocuria marina* BS-15	Sarafin et al. (2014)

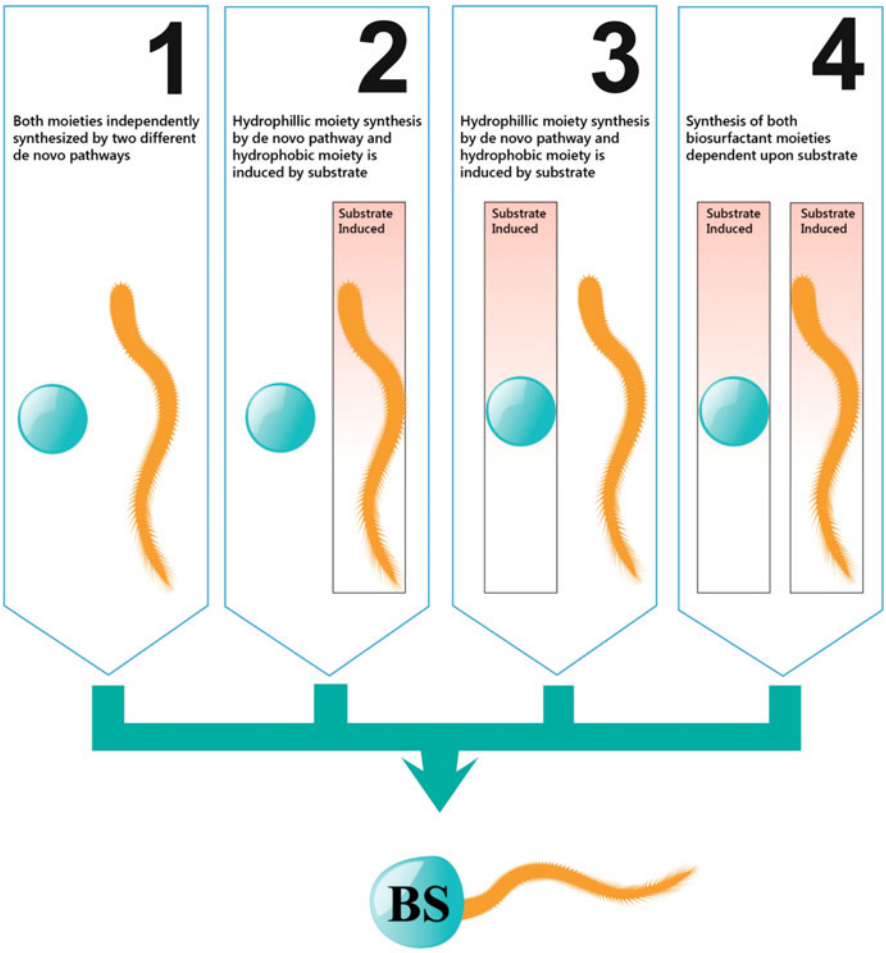

Fig. 1 Four potential pathways for biosurfactant synthesis

microbes synthesize diverse biosurfactant using various pathways involving a variety of enzymes. However, in many cases, regulatory enzymes are the prime enzymes for their synthesis. Therefore, despite structural and functional diversity of biosurfactants, few common features are associated with their synthesis and regulation. According to Syldatk and Wagner (1987), different possibilities which may exist for the synthesis of different biosurfactant moieties and their linkage are explained in Fig. 1.

4 Screening Methods for Selection of Biosurfactant Producers

Extensive and effective screening of biosurfactant-producing microorganisms is required for selecting an efficient producer strain (Maneerat 2005; Satpute et al. 2010). Various isolated microorganisms should be screened properly for the biosurfactant production. Due to functional and structural diversity of biosurfactants, it is very difficult to obtain biosurfactant-producing microorganisms using a single screening method (Satpute et al. 2010). Therefore, multiple screening tests should be used simultaneously to screen out the maximum number of biosurfactant producers among the isolated microorganisms. This also reduces the chance of getting false-positive and false-negative results. Different screening techniques available are direct surface/interfacial tension measurement, oil spreading assay (Morikawa et al. 2000), emulsification capacity assay (Cooper and Goldenberg, 1987), cetyltrimethylammonium bromide agar plate method (Siegmund and Wagner 1991), hemolysis test (Mulligan et al. 1984), drop collapse test (Youssef et al. 2004), and bacterial adhesion to hydrocarbons assay (Rosenberg et al. 1980). Apart from these assays, genes encoding biosurfactants can be directly searched by these molecular techniques. Quick and reliable PCR-based detection of biosurfactant producers has been implemented by different authors to screen microorganisms (mainly *Pseudomonas* and *Bacillus* species) by targeting and amplifying *rhl*AB/*rhl*B gene encoding a rhamnolipid type of biosurfactants (Bodour et al. 2003; Kiran et al. 2010a), *sfp*/*srf*A3 gene encoding surfactin (Hsieh et al. 2004; Simpson et al. 2011; Porob et al. 2013), *lic*A3 gene encoding lichenysin (Simpson et al. 2011), iturin operon which consists of an intergenic sequence between *itu*A and *itu*B genes/*itu*D/*lpa*4 which encodes for iturin (Hsieh et al. 2004; Stankovic et al. 2012), and *fen*D for fengycin (Stankovic et al. 2012). Molecular method-based screening could be used to validate the conventional screening methods with their rapidness, reliability, and efficiency.

5 Industrial Applications of Biosurfactants: A Multifunctional Approach

The functional diversity of biosurfactants which includes emulsification, wetting, cleansing, foaming, dispersion, phase separation, reduction in viscosity, and surface activity results in a broad spectrum of potential applications, being applicable in industries as diverse as petroleum, leather, textile, paper, cosmetic, agriculture, food processing, and pharmaceutical industries (Subasioglu and Cansunar 2008; Randhawa and Rahman 2014). Figure 2 represents the different properties of biosurfactants with their application in various industries. In petroleum industry they could be used for enhanced oil recovery process (Vijayakumar and Saravanan 2015) or for bioremediation purpose (Rahman and Gakpe 2008; Assadi and

Tabatabaee 2010; Pacwa-Płociniczak et al. 2011; Sevilla et al. 2015). Their application during enhanced oil recovery process (microbial enhanced oil recovery, MEOR) results in improving oil drainage into well bore. Biosurfactants cause reduction in interfacial tension as well as oil viscosity and wetting of solid surfaces, thereby stimulating the release of oil entrapped by capillaries (Pacwa-Płociniczak et al. 2011; Randhawa and Rahman 2014). Regarding bioremediation, it is an economical and environmental friendly method for cleaning up oil spills or soils contaminated with petroleum hydrocarbons and dangerous organic compounds like polyaromatic hydrocarbons (Kanaly and Harayama 2000; Panjiar et al. 2013; Sevilla et al. 2015). Bioemulsifiers help in these processes as they reduce the tension at the hydrocarbon-water interface resulting into pseudosolubilization via micelle or vesicle formation. Thus it increases the mobility, bioavailability, and subsequent biodegradation. Moreover, biosurfactant-producing microorganisms may degrade hydrocarbons in both soil and aquatic environment, thus causing more efficient biodegradation (Assadi and Tabatabaee 2010; Pacwa-Płociniczak et al. 2011; Zhang et al. 2012). Although the degradation process is time taking but it is toxin resistant, there is reduction in the risk, labor, and equipment cost. Also it may be employed in areas that cannot be reached easily without excavation, for example, groundwater contaminated with hydrocarbon spills (specifically, petrol spills). They could also be employed for cleaning of oil storage tanks which otherwise involve hazardous, expensive, and time-consuming processes like manual cleaning or solvent washing (Bognolo 1999; Vijayakumar and Saravanan 2015). Their environmental applications also include soil flushing as it enhances solubility of organic or inorganic components (Pacwa-Płociniczak et al. 2011). These properties make them applicable for enhanced remediation of organic contaminants. They are also used for pesticide degradation like hexachlorocyclohexane (Vyas and Murthy 2014).

Biosurfactants partially eliminate the carbon dioxide emissions in the environment, thus playing a crucial role in reducing greenhouse effect (Rahman and Gakpe 2008). Due to their anionic characteristics, they help in metal removal (Mulligan 2009; Gharaei-Fathabad 2011; Pacwa-Płociniczak et al. 2011) or metal sequestration like cadmium II from kaolinite and zinc from Na-feldspar using rhamnolipid biosurfactants (Asci et al. 2007, 2008). Their applications in food-processing industries include their use as emulsifier, solubilizer, wetting agent, foaming agent, thickener, and lubricating agent (Nitschke and Costa 2007; Rahman and Gakpe 2008). They have potential therapeutic and biomedical applications due to their antimicrobial, anticancer, immunomodulatory activities (Makkar and Cameotra 2002; Rodrigues et al. 2006; Abdel-Mawgoud et al. 2010b; Vatsa et al. 2010; Gharaei-Fathabad 2011; Mandal et al. 2013). In cosmetic industries they are used in beauty products as emulsifiers, foaming agents, solubilizers, cleansers, wetting agents, and antimicrobial agents (Mandal et al. 2013). They are more skin compatible as compared to their synthetic counterparts (Williams 2009). Different sophorolipid-based beauty products include shampoos, hair gels, deodorant sticks, and aftershave lotions (Bognolo 1999; Vijayakumar and Saravanan 2015). One of the studies revealed the application of surfactin in dewatering of peat (Cooper et al. 1988). New developments have been made in the area of

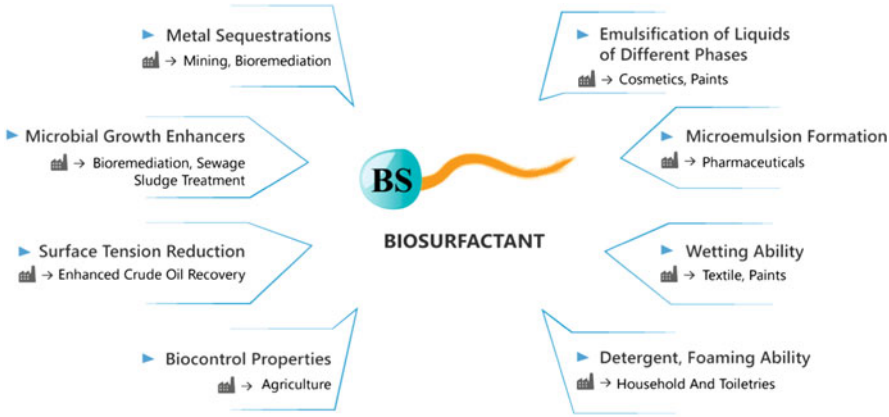

Fig. 2 Properties of biosurfactant with their potential field of application

nanotechnology and biosurfactants. Reddy et al. (2009) reported synthesis of surfactin-stabilized silver nanoparticles. Also studies are available for stabilization of silver nanoparticles by glycolipid biosurfactants produced from *Brevibacterium casei* MSA19 (Kiran et al. 2010a). Its agricultural applications mainly comprise of helping in biocontrol mechanism of microbes such as parasitism along with antibiosis, competition, and hypovirulence (Muthusamy et al. 2008; Ghribi et al. 2011; Mandal et al. 2013). It could also be used for textile dyeing, tanning in leather industry, and pulp treatment in paper industry (Kosaric 2001; Randhawa and Rahman 2014). Emulsion polymerization for paints, paper, and industrial coatings was identified as the second largest market for surfactants (Van Dyke et al. 1991). A polymeric biosurfactant called biodispersan produced by *A. calcoaceticus* A2 has potential use in paint industries (Ron and Rosenberg 2001; Vijayakumar and Saravanan 2015). Reports are available for microbubble technology having a wide scope in molecular imaging, drug delivery, diagnosis of diseases, cost-effective purification of water, and sewage treatment reported to be stabilized by biosurfactants (Xu et al. 2011; Zhang et al. 2011). A recent research on rhamnolipids showed its larvicidal, insecticidal, and repellent activities against *Aedes aegypti*, which further broadens its application areas (Silva et al. 2015).

6 Future Prospects

Advances in modern molecular biology, biochemistry, instrumentation, and computational approaches have created an upsurge in the knowledge of the biosynthesis of bioemulsifiers. Incorporation of molecular techniques and metagenomic approach, apart from conventional methods, for screening could be one way to reach for new and uncultured microbes. This may lead to discovery of novel green bioemulsifiers. The genetic makeup of the producer organisms is one of the vital

factors determining the biosynthesis of biosurfactants. Strain improvement through genetic modifications could be used to significantly improve the yield and quality of produced bioemulsifiers. Furthermore, the information generated can create new opportunities for metabolic engineering to develop more efficient industrial scale production of designer bioemulsifiers, tailored for specific applications.

7 Perspectives

Advancement in white biotechnology which usually involves environment compatible conditions and products along with an increased interest for green chemicals has brought biosurfactants into the research focus during the past few decades. Biosurfactants comprise one of the most versatile groups of compounds having a diverse and broad spectrum of industrial applications. However the bottleneck in its commercialization is its low yield and high production cost. Ever-increasing reports on biosurfactants certainly have increased the target yield, but the desired efficiency is yet to be achieved. There is a need to analyze the overall process, beginning from isolation up to the product recovery, from different perspectives, in order to increase the product yield. Innovative strategies in biotechnology have undoubtedly led to the improved screening and downstream methods which could be employed for efficient recovery of biosurfactants. Moreover, due to the current advances in metabolic engineering, synthetic biology, and instrumentation, target industrial scale production of biosurfactants can be achieved in future.

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Bioproduction of Polyhydroxyalkanoate from Plant Oils

Fakhrul Ikhma Bin Mohd Fadzil and Takeharu Tsuge

Abstract There is a global rise in interest for sustainable biomass-based polymers as promising new material, due to their pronounced advantages such as renewability, biodegradability, and comparable properties to those plastics derived from fossil oil. Several efforts have been made in tackling numerous challenges and drawbacks, especially those focusing on expanding the horizon of bioplastic usage, improvement in polymer properties, processing methods, and cost-effective production. Plant oils and fatty acids derived from them have been used by polymer technologists for the production of a biodegradable polymer, polyhydroxyalkanoates (PHA), using microbial organisms such as *Ralstonia eutropha* and *Escherichia coli*. This chapter will review the literature reporting the perspectives and opportunities in the field of PHA production from plant oils such as corn oil, jatropha oil, palm-based oil, soybean oil, as well as their various fatty acids as feedstocks. The abundance of naturally growing plants that are rich in oils and fats, their relatively low price, and their rich application possibilities make them the most important renewable feedstock in the green plastic industry. This chapter also discusses some introductory concepts about polyhydroxyalkanoates with regard to their engineering applications. Moreover, their properties as well as effective processing techniques, along with the various strategies implemented for their potential commercial applications, have also been presented.

Keywords Plant oils • Polyhydroxyalkanoate (PHA) • Bioproduction • Microbial bioplastics

1 Introduction

An increasing trend in global plastic production and consumption pattern has been observed despite their negative impact toward the environment, as most of plastic products currently used are derived from petroleum, which is a nonrenewable

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source. Fossil-based plastic, accounting for near 280 million tons, were generated in 2011 alone, with an increment of 4% observed in total production till 2016, and are likely to reach 810 million tons by 2050 (Gumel et al. 2013). Greater environmental awareness toward the potential of sustainable plastic materials and fluctuations in oil price, combined with the diminishing fossil fuel resources, recently triggered several efforts, especially from academia, industry, as well as the government to find a better solution for replacing the use of conventional plastics in daily life. Nowadays, green plastics made from renewable resources are attracting worldwide attention as an alternative solution for replacing the use of current petroleum-based plastics. Currently, the market for global bioplastic, which represents 10% to 15% of the total plastic market, is emerging and annual growth of 5% to 8% has been recorded (Lampinen 2010). For these reasons, prospects for developments in commercial production of bio-based plastics are necessary, and the process developed should be reliable, with utilization of cheap renewable resources (López-Cuellar et al. 2011). The modern advancement in biotechnology and recent breakthroughs in engineering practices make it possible to produce bio-based plastics that can be utilized as a green material at various applications. The well-known biodegradable polymers that are well developed and have been commercialized are the polyhydroxyalkanoates (PHAs). PHAs are a group of linear polyesters that are naturally synthesized as intracellular granules by a large number of living microorganisms under limitations of an essential nutrient, along with presence of excess carbon (Poblete-Castro et al. 2012). The greatest advantage that makes PHAs outshine the other bio-based polymers is their ability to degrade naturally without liberating toxic compounds. Thus, PHAs deserve to be recognized as carbon-neutral materials due to their biodegradability under the natural environment by PHA-degrading bacteria. Under appropriate conditions of moisture, oxygen levels, and temperature, biodegradation of PHA results in disintegration of plastics to carbon dioxide and water under aerobic environment, while additionally methane were liberated under anaerobic condition (Ojumu et al. 2004). The biodegradability rate of PHA varies according to their physical and chemical properties. PHAs with lower molecular weight are likely to degrade more easily due to their higher hydrophilicity. However, having a higher melting temperature results in lower susceptibility to disintegration, since it may decrease the enzymatic degradability. Moreover, their chemical structure, which is related to the functional groups, hydrophobic interactions, degree of crystallinity, orientation, as well as morphological properties, is also important (Eubeler et al. 2010). Through the natural degradation phenomena, the carbon continues to disintegrate and is eventually recycled in the environment as carbon dioxide, which eventually gets fixed by photosynthetic organisms. In this regard, PHAs' life cycle is notably regarded as a "zero emission" or "carbon neutral," that is, the net amount of carbon remains constant over time, as no extra carbon is being released into the environment.

Industrial processing of PHA via fermentation uses the combination of renewable feedstock and engineered microorganism, which have been specifically altered to improve PHA yield. The recent enhancement in knowledge in the fields of genetics, metabolic engineering, and enzyme technologies has enabled the improvement of

PHA process reliability, at once increasing the number of potential biopolymers available. In bioprocessing, enzyme is a key engaged in the synthesis of PHAs, with various types of enzymes expressed in different bacteria serving as a biological catalyst. Via metabolic engineering approaches, the specificity of the enzymes toward the intermediates along the biosynthetic pathway can be precisely regulated, allowing for the maximum metabolite flux. Concurrently, during fermentation the extracellular environment comprising of the nutrients in the culture broth provides primary energy source for bacteria growth, wherein the addition of excess carbon promotes the PHA accumulation. In most of studies on PHA production, carbon sources in the form of sugars such as glucose (Kahar et al. 2005), xylose (Le Meur et al. 2012), and fructose (Tan et al. 2014) have been used as feedstock. Although fermentation system using sugars for production of PHAs has been established and optimized in the past, the cost of production still remains high, due to the high cost of input sugar as well as that of the purification process.

Alternatively, the starting raw material for PHA production can be obtained from plant sources, which are renewable and available abundantly. Considering this, their potential has been explored, and they have emerged as well-known sources for sustainable PHA production. Therefore, plastics can now be synthesized in more sustainable way and the cost of production can be lowered, since it involves the utilization of cheap substrates like glycerol (Moita et al. 2014), fatty acids (Liu et al. 2011), and plant oils (Fukui and Doi. 1998; Insomphun et al. 2014). Plant oils are seen as promising renewable resources, being reliable and cheaper way to provide green products, and can potentially substitute other feedstocks for industrial production in the future. Plant oils such as corn oil, jatropha oil, palm-based oil, and soybean oil are available abundantly throughout the year, and this guarantees the supply and availability, thus making them very attractive resources for industrial production. Among these oils, palm oil is classified as a mature and a well-established oil-producing industry (Lam et al. 2009). Countries such as Indonesia, Malaysia, and Thailand are the main producers and exporters for palm oil products, with total production of almost 60 million tons in 2012, thus accounting for about 35% out of global plant oil market share (Hansen et al. 2015). The plant oil primarily comprise of triglycerides made from three fatty acids attached to a glycerol molecule. Moreover, oleochemical industry generates a large amount of fatty acids and glycerol as a by-product, both of which are readily usable as a feedstock for microbial PHA production.

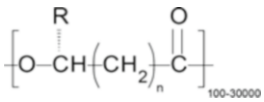
Process economics upon utilizing plant oils is even better when compared with the sugar-based substrate. Having higher carbon content makes them a practical choice and cost-effective resources for large-scale fermentation process, thereby enabling a higher PHA accumulation. Yield of PHA produced from plant oils is reported to be around two times higher (extending to 1 g of PHA per g of plant oils) than yield of PHA obtained by utilizing sugars (Tsuge 2002). Production from glucose only allows for yield of 0.3 to 0.4 g P(3HB)/g glucose (Akiyama et al. 2003) pertaining to low PHA yield. In contrast, the yield coefficient obtained from palm oil bioproduction was reported at 0.84 g P(3HB)/g palm oil (Budde et al. 2011).

In the last two decades, extensive research and development on PHAs with improved mechanical and physical properties have been performed for its greater applications. Metabolically engineered *Escherichia coli* has been studied extensively and has shown an exceptional versatility for PHA biosynthesis. It possesses several advantages, including ability to grow using inexpensive substrate, accumulation of higher PHA content (Wang and Lee 1997), capability of producing PHAs with higher molecular weight than native PHA-synthesizing bacteria (Kusaka et al. 1997), faster growth (to reach a high density, which is important for achieving high productivity) (Kahar et al. 2005), deficiency in an intracellular depolymerases (which prevents the degradation of stored PHAs) (Cai et al. 2009), and relative ease of purification.

In addition, well-known PHAs producing bacteria such as *Ralstonia eutropha* (also known as *Alcaligenes eutropha*, *Wautersia eutropha*, and *Cupriavidus necator*) and *Pseudomonas putida* have been shown to efficiently accumulate high levels of PHAs using plant oils and fatty acids as their energy source. With the high demand for PHAs, ongoing supports, and perhaps the well carried out research work, which has driven the improvement in properties as well as reduction in cost, it can be hoped that green plastic industry will continue to grow with unlimited applications.

2 Polyhydroxyalkanoate (PHA): Types, Structure, and Properties

Polyhydroxyalkanoates (PHAs) are a group of linear polyesters that are naturally produced by bacterial fermentation for carbon and energy storage. PHAs consist of carbon, hydrogen, and oxygen, with each monomer unit harboring a side chain, R group (Fig. 1). The nomenclature and carbon number for PHAs are given according to their functional alkyl, R group. PHAs are produced intracellularly by bacteria and are stored as inclusion bodies in its cytoplasm. Numerous bacteria, both Gram negative and Gram positive, can synthesize PHA polymers and store it as intracellular inclusion bodies. These include *Pseudomonas*, *Ralstonia*, *Cupriavidus*, *Aeromonas*, *Bacillus*, *Alcaligenes*, *Enterobacter*, and *Rhodobacter* along with some of the Halobacteriaceae (Han et al. 2015; Tsuge et al. 2015) and Cyanobacteria (Yan et al. 2010). PHAs can be further classified into two types based on the carbon number in their monomeric units: the short-chain length (scl) PHAs containing 5 or less carbons and the medium-chain length (mcl) PHAs having 6–14 carbons. Moreover, there are also PHA copolymers such as scl copolymers [e.g., poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), P(3HB-*co*-4HB); poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV)] and mcl copolymers [e.g., poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate), P(3HHx-*co*-3HO); poly(3-hydroxhexanoate-*co*-3-hydroxydecanoate), P(3HO-*co*-3HD)] as well as copolymers incorporating both scl and mcl monomers [e.g., poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), P(3HB-*co*-3HHx)]. The thermal and mechanical properties of PHAs depend upon the copolymer composition. Therefore,



	Side chain alkyl group	PHAs
n = 1	R = hydrogen	3-hydroxypropionate = 3HP*
	R = methyl	3-hydroxybutyrate = 3HB
	R = ethyl	3-hydroxyvalerate = 3HV
	R = propyl	3-hydroxyhexanoate = 3HHx
	R = isopropyl	3-hydroxy-4-methyvalerate = 3H4MV
	R = pentyl	3-hydroxyoctanoate = 3HO
	R = heptyl	3-hydroxydecanoate = 3HD
	R = nonyl	3-hydroxydodecanoate = 3HDD
n = 2	R = hydrogen	4-hydroxybutyrate = 4HB*
n = 3	R = hydrogen	5-hydroxyvalerate = 5HV*

* Achiral monomer.

Fig. 1 General structure of polyhydroxyalkanoates

by knowing the factors that affect the composition of PHAs copolymer during their synthesis, exclusive PHAs with desired attributes and characteristics can be produced. For example, heteropolymers produced by varying their comonomer composition may be changed in the degree of flexibility, either becoming hard and rigid or having elastic properties with rubbery performance. Among various types of biopolymers, P (3HB) was the first and foremost PHA to be discovered and, thus, has been most extensively worked upon as a pioneer model for PHA development. P(3HB) has inferior properties, being relatively brittle, rigid, stiff, and exhibits highly crystalline morphology. Having a melting temperature at 177 °C, which is near to its thermal decomposition temperature beginning significant molecular weight decrease, further prevents its applications due to higher degree of process complexity. At around 200 °C, P(3HB) begins to decompose and reduce its molecular weight, thus making its processing via injection molding extremely difficult.

However, P(3HB) has features and functionalities comparable to the conventional plastics manufactured from petroleum such as polyethylene and polypropylene. The P(3HB) pallets can be further processed by extrusion, injection molding, electrospinning, production of fibers, or even blending with other polymers to form heteropolymers with advanced properties. The mechanical properties of P(3HB) define how it will react with external forces. Having Young's modulus (3.5 GPa), tensile strength (40 MPa), and elongation to break (6%) (Table 1) does not give a lot of advantage for its various applications, since P(3HB) is unstretchable and tends to break easily. In terms of its weight-average molecular weight (M_w), native-PHAs synthesizing bacteria commonly produce P(3HB) between 1×10^4 to 3×10^6 (Khanna and Srivastava 2005a). However, extremely high molecular weight P (3HB), up to 20×10^6 (Kusaka et al. 1997), can be produced by using recombinant *E. coli*. Despite that fact that P(3HB) has brittle features and exhibits poor elastic behavior, there is still a huge scope of improvement in its properties. In a

different study, further improvement has been achieved by obtaining ultrahigh molecular weight P(3HB) [UHMW-P(3HB)]. This lead to an increase of 33-fold in the tensile strength (of up to 1.32 GPa) as compared to the lower molecular weight P(3HB), making it stronger and tougher. This was accomplished by processing the fiber by combining cold-drawing and two-step drawing procedures at room temperature (Iwata et al. 2004). Similarly, another study successfully produced UHMW-P(3HB) with M_w greater than 3×10^6. As described previously, different methods of polymer processing via combination of annealing and drawing treatment (Aoyagi et al. 2003) resulted in formation of strong P(3HB) fibers with Young's modulus (2.94 GPa), tensile strength (388 MPa), and elongation to break (25%). Eventually, the supramolecular weight P(3HB), with extremely high molecular weight (20×10^6), was produced from recombinant *E. coli* under optimal growing condition and efficient processing (Choi and Lee 2004). All of these advanced features and superior properties are better suited over the low molecular weight P(3HB), in fabricating highly strong fibers or films especially for medical application and food packaging industry.

Despite of the non-inferior properties of PHAs, incorporation of existing polymers with other hydroxyalkanoates monomer components to form heteropolymers could also enhance their physical properties like crystallinity, melting point, stiffness, and brittleness, which are necessary for transcending the boundary limits in their current applications. Because of the ongoing research that has been carried out till now, it is hoped that the anticipated commercialization can be realized in a couple of years to fulfill the growing demands for green polymers. Looking back to the history of PHAs, the commercialization of first PHA can be traced back to 1980s. Commercial production of PHA was established in the United Kingdom by Imperial Chemical Industries (ICI), and P(3HB) produced was traded with the brand name Biopol™. Today, a wide range of selection for different P(3HB)-based grades are commercially available and are produced by various companies notably Aonilex® by Kaneka Co. Ltd. (Japan), Mirel™ produced by Telles (USA), Biocycle® from PHB Industrial Company (Brazil), Biomer® by Biomer Inc. (Germany), and Enmat® by Chinese bioindustry firm, Tianan Biologic, Ningbo.

In the P(3HB) biosynthesis, propionate was supplemented during the growth of a mutant *Ralstonia eutropha*, for producing a random copolymer of poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] (Shang et al. 2004). The properties of PHBV formed are greatly influence by the percentage of 3-hydroxyvalerate (3HV) present in the heteropolymers moiety. Increasing the mole fraction of 3HV from 0 to 25% resulted in decreasing of the Young's modulus, tensile strength, and melting point (Table 1). It has been reported that higher 3HV fractions are observed in low crystallinity, while other noteworthy features were found to increase, making the polymer produced more flexible and ductile (Nduko et al. 2012). Bearing in mind that highly crystalline degree of copolymers are

Table 1 PHA types with their physical and mechanical properties

PHA type	Physical and mechanical properties					Reference
	Melting temperature T_m (°C)	Glass transition temperature T_g (°C)	Tensile strength (MPa)	Young's modulus (MPa)	Elongation to break (%)	
P(3HB)	180	4	40	3500	5	Fukui and Doi (1998)
P(3HB-*co*-3HV)						Doi (1990)
3 mol% 3HV	170	8	38	2900	–	
9 mol% 3HV	162	6	37	1900	–	
14 mol% 3HV	150	4	35	1500	–	
20 mol% 3HV	145	–	32	1200	–	
25 mol% 3HV	137	–	30	700	–	
P(3HB-*co*-4HB)						Saito and Doi (1994), Saito et al. (1996)
3 mol% 4HB	166	–	28	–	–	
10 mol% 4HB	159	–	24	–	400	
16 mol% 4HB	130	−7	26	–	444	
64 mol% 4HB	50	−35	17	30	591	
78 mol% 4HB	49	−37	42	24	1120	
90 mol% 4HB	50	−42	65	100	1080	
P(4HB)	53	−48	104	149	1000	
P(3HB-*co*-3HHx)						Doi et al. (1995)
10 mol% 3HHx	127	−1	21	–	400	
15 mol% 3HHx	115	0	23	–	760	
17 mol% 3HHx	120	−2	20	–	850	
P (3HB-*co*-3H4MV)						Tanadchangsaeng et al. (2009)
9 mol% 3H4MV	146	−2	22	700	30	
16 mol% 3H4MV	133	−2	17	390	330	
22 mol% 3H4MV	118	−2	–	250	–	
38 mol% 3H4MV	113	−4	–	–	–	

(continued)

Table 1 (continued)

PHA type	Physical and mechanical properties					
	Melting temperature T_m (°C)	Glass transition temperature T_g (°C)	Tensile strength (MPa)	Young's modulus (MPa)	Elongation to break (%)	Reference
P(3HO)	45	−36	–	11	–	Rai et al. (2011)
P(3HD)	72	−37	12	20	313	Liu et al. (2011)
P(15% 3HD-*co*-85% 3HDD)	78	−32	5	103	88	

desired, improvement of the crystallinity by introducing boron nitride as a nucleating agent has been demonstrated, which further enhanced the crystallization rates (Wang et al. 2010). Tanaka and co-workers successfully produced fibers with extremely high tensile strength (up to 1.3 GPa) by conducting one-step drawing procedure followed by growing of small crystal nuclei using isothermal crystallization near glass transition temperature (Tanaka et al. 2006). As discussed earlier, P(3HB) begins to decompose near its melting temperature; therefore reduction in melting point by incorporation of higher 3HV fraction significantly improves its stability and enables the copolymer to be processed without degradation. However, the heteropolymers thus formed still have several limitations such as a slow crystallization rate and tendency for secondary crystallization during storage (Biddlestone et al. 1996). Apparently, P(3HB) and their copolymers are still being pursued despite their shortage to meet the diverse needs by various industries.

It, however, seems that another type of commercially important PHA having appealing features is becoming a main rival to P(3HB). This is poly(4-hydroxybutyrate) [P(4HB)], which is exceptionally flexible and strong, thus making it a perfect biomaterials for medical and pharmaceutical application. P(4HB) is generally described as a stretchable and tough thermoplastic, having a remarkable tensile strength that is almost equivalent with ultrahigh molecular weight polyethylene (Engelberg and Kohn 1991). It is more stretchable compared to the other homopolymer PHAs, having a high elongation to break of around 1000% enabling P(4HB) to be stretched ten times from its original length. Additionally, it has been regarded as a biocompatible material and is completely safe for in vivo application, since the biodegradation of P(4HB) yields 4-hydroxybutyric acid, a metabolite that is naturally produced and present in the human body cells. For clinical application, P(4HB) was approved by the US Food and Drug Administration (FDA) as an absorbable suture under brand of TephaFLEX®.

With successful development of P(4HB), more PHA-based biomaterials with broader range of properties are expected to be ready for extended use. Copolymerization of 4HB with 3HB, for instance, can further enhance its mechanical properties. P(3HB-*co*-4HB) fibers produced are remarkably strong, and their stretchability can be varied according to their monomer composition. Increasing 4HB fractions from 16% to 90% turned the neat P(3HB) from hard crystalline plastics to elastic rubber type materials (Saito et al. 1996). Furthermore, besides their truly exceptional characteristic, these heteropolymers also show degradation under various environmental conditions (Doi et al. 1990, 1992), therefore enabling them to be suitable for use in any application that requires a specific environment.

In the present decade, a great interest has emerged in the study of mcl-PHAs. Medium-chain-length PHAs synthesized from bacteria primarily consist of 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), and 3-hydroxydodecanote (3HDD) (Ma et al. 2009). When compared with other scl-PHAs, mcl-PHAs are generally amorphous and very elastomeric due to their low degree of crystallinity, along with a high elongation to break. A distinguishing feature of mcl-PHAs from scl-PHAs is their low melting temperatures with broad melting ranges (40–60 °C), low glass transition temperatures (around

−40 °C), low crystallinity (X_c lower than 40%), and low degrees of polymerization (M_w typically less than 10^4 g/mol) (Muhr et al. 2013). All these physical properties and features of mcl-PHAs make them considerably good for commercialization. However, they exhibit adhesive properties and are too sticky to be processed conveniently, thus limiting their further applications.

Much effort has been carried out in exploiting this new promising class of PHAs. A type of mcl-PHA, named as P(3HHx), had been synthesized using *Pseudomonas putida* KTHH03. Upon growth on hexanoate as a carbon source, polymers with an amorphous and sticky features were produced without any development in crystallinity (Wang and Lee 1997). On the other hand, another type of mcl-PHA, P(3HO), was successfully synthesized and has a semicrystalline structure with lower Young's modulus (11.4 MPa). This homopolymer film produced from *Pseudomonas mendocina* has been found to have smoother surface than other PHAs such as P(3HB) (Rai et al. 2011). The resulting polymer produced has a flexible and elastic characteristic.

Furthermore, a study conducted by Liu et al. (2011) showed for the first time that inhibiting *β*-oxidation in *P. putida* KTQQ20 resulted in production of P(3HD) homopolymer and their copolymers containing 3HDD or/and 3-hydroxytetradecanoate (3HTD) with an amorphous morphology. All physical properties of mcl-PHAs synthesized upon growth in decanoate are presented in Table 1. Improvement of mechanical properties of the P(3HD-*co*-3HDD) copolymer was observed upon incorporation of 85 mol% of 3HDD monomers, resulting in the increase of its tensile strength and elongation to break, whereas decreasing the Young's modulus. These changes are related to the monomer homogeneity.

Of even greater interest is P(3HB-*co*-3HHx), a special heteropolymer having of both scl monomer and mcl-monomer in its backbone. It has been reported that incorporation of 10 mol% 3HHx fraction into the neat 3HB structure greatly improves the thermal processability and physical properties of P(3HB). Through incorporation of 3HHx units at 10 mol% into the 3HB polymer constituent, the elongation to break significantly improved, hence increasing the polymer flexibility compared to the neat P(3HB) (Doi et al. 1995). Moreover, a fixed 3HHx monomer composition at 5 mol% was frequently obtained, regardless of the types, concentration, and sources of plant oils used as a substrate (Loo et al. 2005).

A unique heteropolymer with branched monomer in the side chain, termed 3-hydroxy-4-methylvalerate (3H4MV), is appealing for improving the properties of neat P(3HB). Upon supplementation of 4-methylvalerate (4MV) as 3H4MV precursor, the incorporated 3H4MV units are able to enhance the brittle and stiff P(3HB), when introduced to the polymer moiety at about 11 mol% 3H4MV. The resulting copolymer, P(3HB-*co*-3H4MV), featured lower melting temperature than neat P(3HB), suggesting that 3H4MV contributed to beneficial effect on improving the thermal properties of P(3HB). Moreover, an increase in the elongation to break was reported upon incorporation of 3H4MV fraction from 9 mol% to 16 mol%, thus successfully enhancing the flexibility (Tanadchangsaeng et al. 2009).

These are among some of the representative works on combination of genetic manipulation and bioprocessing approaches for production of PHAs of desired composition. Similar further developments are expected in future, since the trend of mcl-PHAs use is gaining more popularity, particularly in the field of medical applications, where flexible biopolymers are critically needed.

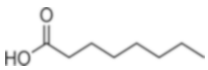
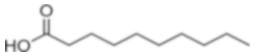
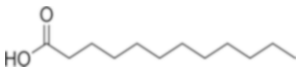
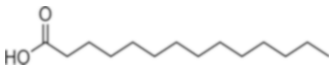
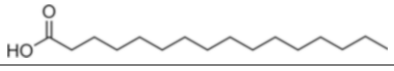
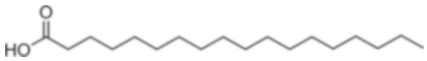
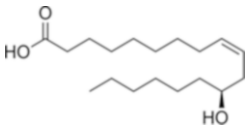
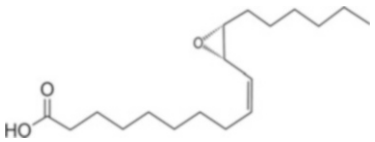
3 Microbial Bioplastic Production from Plant Oil

Plant oils are important commodity and sustainable resources obtained from agricultural activities. They are available abundantly at low price and chemically consist of triglycerides formed between a glycerol molecule and fatty acids of various lengths (Table 2). In recent times, numerous projects for PHA production using plant oils were carried out due to low material costs, and this lead to higher yield of PHAs. At first, the feasibility study for P(3HB) production using plant oil was performed using *Alcaligenes* species (Akiyama et al. 1992). This new isolated strain was cultivated on medium enriched with various plant oils such as corn, olive, palm, rapeseed, and soybean oils as well as *n*-alkanoic acids. Supplementation of *n*-alkanoates with even carbon numbers generated P(3HB) homopolymer, whereas addition of *n*-alkanoates with odd carbon numbers surprisingly produced P(3HB-*co*-3HV) copolymers. Via a small-scale production, relatively high biomass concentration at 3.11 g/L cell dry weight (CDW) with 47 wt% P(3HB) content had been achieved, indicating that plant oils are suitable and have a great potential to be used as a feedstock for producing PHA.

Further improvement was achieved with wild-type *Ralstonia eutropha* and a PHA-negative mutant of *R. eutropha* PHB^{-}4 (Saika et al. 2015) harboring a PHA synthase gene, $phaC_{Ac}$, from *Aeromonas caviae*. While in the native strain, an accumulation of P(3HB) homopolymer has been observed, on the contrary, in the mutant strain, random copolymers of P(3HB-*co*-3HHx) with 80 wt. % PHA content were produced during the stationary phase (Fukui and Doi 1998). In addition, Fukui and Doi demonstrated that the native strain of *R. eutropha* H16 was capable of accumulating PHA with similar trends, with up to 80 wt.% PHA content obtained from dry cells when grown on corn oil, olive oil, palm oil, and oleic acid.

The wild-type *Ralstonia eutropha* synthesizes PHAs that consists of hydroxyalkanoic acids with short carbon chain length, as 3HB is the major constituent in the polymer chain. In *R. eutropha*, intermediates and products from fatty acid metabolism are finely connected to PHA metabolic pathways. Both wild-type and mutant strains of *R. eutropha* can synthesize PHA precursors, (*R*)-3-hyroxyacyl-CoA molecules either from β-oxidation or fatty acid biosynthesis (Mifune et al. 2008). In the case of β-oxidation intermediates, overexpression of *phaJ* gene encoding for (*R*)-specific enoyl-CoA hydratase lead to the formation of (*R*)-3-hyroxyacyl-CoA from 2-*trans*-enoyl-CoA, by catalytic reaction. In different path, via fatty acid biosynthesis, *phaG* gene is overexpressed, which encodes for 3-hydroxyacly-ACP: CoA transferase and catalyzes the formation of (*R*)-3-hyroxyacyl-CoA from

Table 2 Important fatty acids with their chemical formula and structure

Fatty acid	Chemical formula	Structure
Caprylic (octanoic)	$C_8H_{16}O_2$	
Capric (decanoic)	$C_{10}H_{20}O_2$	
Lauric (dodecanoic)	$C_{12}H_{24}O_2$	
Myristic (tetradecanoic)	$C_{14}H_{28}O_2$	
Palmitic (hexadecanoic)	$C_{16}H_{32}O_2$	
Stearic (octadecanoic)	$C_{18}H_{36}O_2$	
Oleic	$C_{18}H_{34}O_2$	
Linoleic	$C_{18}H_{32}O_2$	
α-Linolenic	$C_{18}H_{30}O_2$	
α-Eleostearic	$C_{18}H_{30}O_2$	
Ricinoleic	$C_{18}H_{34}O_3$	
Vernolic	$C_{18}H_{32}O_3$	

(*R*)-3-hyroxyacyl-ACP. Both genes used in these metabolic engineering have originated from *Pseudomonas* species (Davis et al. 2008; Sato et al. 2011). Through metabolic engineering, PHA production could be enhanced, thus making the process economical. It has been reported that overexpression of either one of these two genes in the biosynthesis of P(3HB-*co*-3HHx) by *R. eutropha* grown in soybean oil as the sole carbon source is capable of producing higher 3HHx fraction (Kawashima

et al. 2012). Moreover, there are several other strategies in metabolic engineering that can be performed, such as replacing the native PHA synthase involved in PHA biosynthesis. Since the native PHA synthase (PhaC) in *R. eutropha* is specific for scl monomers, replacing PHA synthase with enzymes with wide substrate specificity can incorporate scl and mcl monomers into the heteropolymers moiety, which is necessary for obtaining desired PHAs with different monomeric compositions. For example, through insertion of *phaJ* gene from *Aeromonas caviae* and overexpression of PHA synthase from *Rhodococcus aetherivorans* I24 (Wong et al. 2012) in copolymer production, mutant *R. eutropha* was able to produce higher 3HHx level (up to 9.9 mol%) with 79 wt.% PHA.

Although polymer content obtained in previous works are considerably higher, a high cell density culture and PHA yield must be established as a consideration for the reproducibility in the industrial process. Several studies have reported that high CDW can be obtained from utilization of plant oils. The well-known soybean oil has been tested in the bioproduction of PHAs using a wild-type *R. eutropha* H16 and a mutated strain PHB^{-}4/pJRDEE32d13, performed well both at flask scale and using jar fermenter (Kahar et al. 2004). During the fermentation, soybean oil and ammonium chloride were supplied continuously and maintained at concentration of 20 g/L and 4 g/L, respectively, by pulse feeding system. It is important to keep both carbon and nitrogen sources at optimum concentrations to ensure higher cellular metabolism and PHA accumulation. Introducing foreign genes for manipulating the metabolic pathway often required the addition of antibiotics to ensure the plasmid stability. During the fermentation, antibiotic was supplemented to maintain the stability of the plasmid carrying the $phaC_{Ac}$ gene, which encodes for a broad substrate specificity of PHA synthase. However, antibiotics are not practical to be used for large-scale production, as they may contribute toward the higher production cost. The fermentation achieved very high CDW, in the range between 118 and 126 g/L with 72–76 wt.% P(3HB) content upon growth with wild-type strain H16. A small fraction of copolymer content with 5 mol% 3HHx was synthesized from recombinant strain, capable of producing 128–138 g/L CDW with 71–74 wt.% PHA content. This provides convincing result for the reproducibility of PHA produce from soybean oil by maintaining carbon and nitrogen level at optimal concentration, giving yield at 0.72–0.76 g PHA per g soybean oil. It was also reported that under phosphate limiting condition, only 95.5 g/L of CDW was produced, which was much lower than without limitation. In other study, *R. eutropha* KCTC 2662 cultures were grown in batch and fed-batch fermentations, which were supplemented with soybean oil for P(3HB) production. High cell concentration of 15–32 g/L was obtained with 78–83 wt. % of PHA and yield of 0.80–0.82 g P(3HB) per g soybean oil (Park and Kim 2011). Soybean oil primarily contains distributions of monounsaturated, polyunsaturated, and saturated fatty acids comprising of approximately 53% of linoleic, 23% of oleic, 11% of palmitic, 8% of linolenic, and 4% of stearic acid (Table 3). Secretion of microbial lipase hydrolyzed the fats, forming triacylglycerol and fatty acids that are released into the culture broth, and eventually getting utilized for growth and accumulation of PHA. This strain effectively consumes soybean oil at optimum carbon to nitrogen ratio

Table 3 Plant oils with their fatty acid composition

Plant oil	Fatty acid composition (%)					Reference
	Palmitic	Stearic	Oleic	Linoleic	Linolenic	
Castor	1.0	1.0	3.0	4.2	0.3	Gui et al. (2008)
Jatropha	14.2	7.0	44.7	32.8	–	Akbar et al. (2009)
Palm	42.8	4.2	40.5	10.1	–	Xia and Larock (2010)
Soybean	11.0	4.0	23.4	53.3	7.8	
Corn	10.9	2.0	25.4	59.6	1.2	
Sunflower	5.2	2.7	37.2	53.8	1.0	
Rapeseed	4.0	2.0	56.0	26.0	10.0	
Cottonseed	21.6	2.6	18.6	54.4	0.7	

(C/N) of 20, with $(NH_4)_2SO_4$ used as a nitrogen source to attain the maximum cell concentration and PHA accumulation. However, the C/N ratio strongly affects the culture growth, with a reduction in cell concentration and PHA yield observed at higher C/N ratios.

In more recent studies, biosynthesis of P(3HB) with even higher yield of 0.85 g per g soybean oil has been reported (Pradella et al. 2012) by using *Ralstonia eutropha* (also known as *Cupriavidus necator*). Pulse feeding strategies have been implemented to overcome carbon limitation caused by depletion of soybean oil after 12 hours of cultivation. As a result, high cell concentration of 83 g/L was attained by addition of 40 g/L soybean oil with a pulse fed at the same initial concentration of soybean oil. The P(3HB) content obtained was at 81 wt.% from its CDW, with a yield of 0.85 g P(3HB) per g of soybean oil utilized (Table 4).

Recently there has been a keen interest in the utilization of oil from the palm industry to produce PHAs. In an effort to replace the conventional plastics, palm-based oil resources have been used in producing numerous types of PHAs. An exploratory approach on PHA production from palm oil began around two decades ago, when Majid and colleagues attempted to synthesize P(3HB) from palm-based cooking oil by using newly identified *Alcaligenes* species, named as *Alcaligenes* sp. AK 201 (Majid et al. 1994). Two g/L P(3HB) was successfully obtained resulting from supplementation of the growing medium with 3 g/L of palm oil. Further studies were carried out by the same group using the locally isolated *Erwinia* sp. USMI-20 strain, which was cultivated in batch fermentation with various palm oil-based substrates, among them are crude palm oil (CPO) and palm kernel oil (PKO). P(3HB) content with 46 wt.% from 3.6 g/L CDW was obtained by supplementation of 4.6 g/L palm oil at initial stage (Majid et al. 1999).

In recent times, a different group trying to grow *Ralstonia eutropha* on palm kernel oil was able to produce 7.9 g/L CDW with nearly 80 wt.% accumulated P (3HB) (Bhubalan et al. 2008). This work utilized 3HV precursors in the form of sodium propionate and sodium valerate for the biosynthesis of new heteropolymer termed as P(3HB-*co*-3HV-*co*-3HHx) with remarkable features. Also by utilizing PKO, Lee and colleagues obtained 7.5 g/L CDW with around 90 wt.% of P (3HB-*co*-3HV) content using *R. eutropha* H16. P(3HB-*co*-3HV) with different

Table 4 High cell density fermentation for production of PHAs from plant oils using various native and engineered strains

Strains (PHA synthase expressed)	Carbon Source		PHAs	CDW (g/L)	Polymer content (wt. %)	Yield (g-PHA/g-plant oil)	Reference
	Plant oil	Co-source					
Ralstonia eutropha H16, *Alcaligenes eutrophus* H16, *Cupriavidus necator* H16	Rapeseed oil, Corn oil	Propionate	P(3HB-*co*-3HV)	140–204	64–72	0.64–0.72	Naylor and Wood (1999)
	Soybean oil	–	P(3HB)	118–126	72–76	0.72–0.76	Kahar et al. (2004)
	Waste rapeseed oil	Propanol	P(3HB-*co*-3HV)	138	76	0.83	Obruca et al. (2010)
Ralstonia eutropha KCTC 2662	Soybean oil	–	P(3HB)	15–32	78–83	0.80–0.82	Park and Kim (2011)
	Soybean oil	γ-Butyrolactone	P(3HB-*co*-4HB)	10–21	83–95	0.45–0.56	
Ralstonia eutropha PHB^{-}4 (*Aeromonas caviae*)	Soybean oil	–	P(3HB-*co*-3HHx)	128–138	71–74	0.72–0.74	Kahar et al. (2004)
Ralstonia eutropha H16Δ*phaC* (*Rhodococcus aetherivorans*)	Palm oil	–	P(3HB-*co*-3HHx)	139	74	0.63	Riedel et al. (2012)
Ralstonia eutropha H16Δ*phaC* (*Aeromonas caviae*)	Soybean oil	–	P(3HB-*co*-3HHx)	52	66	–	Insomphum et al. (2014)
	Palm kernel oil	–	P(3HB-*co*-3HHx)	165–171	73–78	–	Sato et al. (2015)
	Palm kernel oil	Butyrate	P(3HB-*co*-3HHx)	153–175	74–79	–	

3HV molar fractions, produced from a combination of palm kernel oils, and 3HV precursors were reported to have a high range of M_w between 1.4×10^6 and 3.1×10^6 (Lee et al. 2008). The 3HV monomer fractions can be adjusted accordingly in the range of 0–23 mol% by varying the cultivation conditions such as amount of 3HV precursor added, nitrogen source, and the initial pH. Later, P(3HB-*co*-3HV) and P(3HB-*co*-4HB) copolymers were synthesized from CPKO with addition of 3HV or 4HB precursor (Kek et al. 2010). Up to 31 mol% of 3HV can be acquired by varying the 3HV addition time. Through supplementation of 4HB precursor, 67 wt.% of P(3HB-*co*-4HB) content, accounting for around 5 mol% of 4HB, was produced, irrespective of the precursor addition time applied.

Furthermore, upscaling of the PHA copolymer production from palm oil was demonstrated using fed-batch fermentation, resulting in higher cellular production. A mutant PHA-producing strain, *R. eutropha* Re2058/pCB113 harboring $phaC2_{Ra}$ gene from *Rhodococcus aetherivorans* and $phaJ1_{Pa}$ gene from *Pseudomonas aeruginosa*, was grown using fed-batch fermentation mode, whereby PHA accumulation was triggered under nitrogen limiting condition (Riedel et al. 2012). Another important parameter for promoting higher cell growth and PHA yield is the type of nitrogen source supplemented. It has been found that urea was the most preferred nitrogen source, over NH_4OH and NH_4Cl feeding for *R. eutropha* in producing PHA. Supplementation of urea as initial nitrogen source contributed to produce 48% more PHA (Khanna and Srivastava 2005b). This can be explained by the fact that the CO_2 produced after the nitrogen consumption from urea can further serve as a secondary carbon source and can potentially enhance the cell growth. Thus, higher process productivity was established by urea addition resulting in up to 139 g/L CDW with 74 wt. % of PHA that contain 19 mol% 3HHx (Riedel et al. 2012).

It can be emphasized here that the presence of the plasmid addiction system in Re2058/pCB113 strain for maintaining plasmid stability was sturdy for high cell density fermentations, which are capable of producing a maximum CDW of up to 140 g/L (Riedel et al. 2012). Therefore, an antibiotic addition to the media for plasmid maintenance is unnecessary, since it may increase the cost of production, especially for large-scale production. Recently, Sato and colleagues have successfully constructed a new stable plasmid vector (pCUP3) for effective production of P(3HB-*co*-3HHx) using *Ralstonia eutropha* H16 as the host strain, without the requirement for any antibiotic (Sato et al. 2013).

Another available feedstock derived from palm industry is the crude palm kernel oil (CPKO), obtained from palm kernel extraction. Utilizing CPKO, P(3HB-*co*-3HHx) containing higher 3HHx monomer fraction with softer texture and higher flexibility was synthesized by recombinant strain *R. eutropha* Re2160/pCB113 (Wong et al. 2012), which harbors wild type PHA synthase from *R. aetherivorans*. A maximum production of copolymer with 68 mol% of 3HHx was obtained once 2.5 g/L of CPKO was fed during cultivation. In contrast, further increment of CPKO concentrations in the range of 5.0 g/L to 20 g/L had no significant effect toward the 3HHx monomer composition, whereas the CDW was reduced by about 2.5-fold at high CPKO concentration.

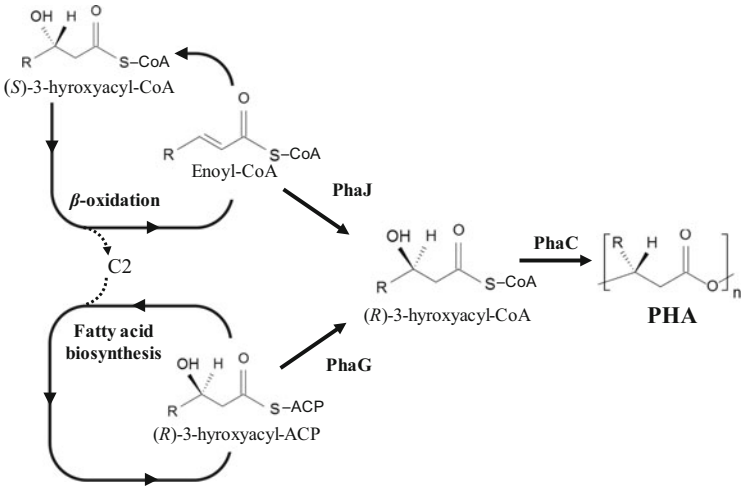


Fig. 2 Interlinkages between fatty acid metabolic pathways involved in PHA biosynthesis in wild-type and mutant *R. eutropha*. (*R*)-specific enoyl-CoA hydratase (PhaJ) catalyzes the formation of (*R*)-hydroxyacyl-CoA, a PHA precursor from enoyl-CoA, from *β*-oxidation cycle intermediates. (*R*)-3-hyroxyacyl-CoA can also be synthesized from fatty acid biosynthesis intermediates by the action of 3-hydroxyacyl-ACP:CoA transferase (PhaG)

Fatty acids have also been in focus for the production of PHA due to their competitive price and abundant availability in nature. In the past, production of mcl-PHAs has been demonstrated from various fatty acids with different chain lengths. Biosynthesis of mcl-PHAs by *Pseudomonas* spp. is closely related to fatty acid *β*-oxidation and de novo fatty acid biosynthesis (Fig. 2); hence alteration of the metabolic pathway has been performed as a strategy to increase PHA accumulation (Cai et al. 2009). Ouyang and colleagues successfully produced mcl-PHA consisting 3HDD from engineered *P. putida* KT2442 strain with a weakened *β*-oxidation pathway. Two important enzymes in the last two steps of *β*-oxidation has been identified, a 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase specified by *fadA* and *fadB* genes, respectively (Ouyang et al. 2007), and both genes were knocked out in attempting to weaken the *β*-oxidation pathway.

Production of mcl-PHA by a mutant strain *P. putida* KTOY06 with dodecanoate supplementation as a sole carbon source eventually accumulated 84 wt.% PHA with higher 3HDD fractions of over 40 mol%. In addition, PHA accumulation can be further enhanced by overexpression of two key enzymes, (*R*)-specific enoyl-CoA hydratase (PhaJ) (Tsuge et al. 2003) and 3-ketoacyl-CoA reductase (FabG) (Nomura et al. 2005), in a functional *β*-oxidation pathway. The strategy, when employed, leads toward buildup of (*R*)-3-hydroxyacyl-CoA, an intermediate resulting from *β*-oxidation which becomes a substrate for PHA synthase (PhaC), the key enzyme for synthesis of PHA. Moreover, deletion of *phaG* gene that encodes 3-hydroxyacyl-CoA-acyl carrier protein transferase (ACP) resulted in weakening the fatty acid *β*-oxidation activity (Liu et al. 2011). Apart from that,

formation of PHA monomers and their structures can also be altered by manipulating the associated genes in fatty acid β-oxidation through genetic engineering. Thereby, inactivation of genes engaged in β-oxidation such as *fadB* and *fadA* may increase the PHA copolymer production (Ouyang et al. 2007). Deletion of *fadA* genes partially decoupled the interconversion between TCA cycle and fatty acid β-oxidation; thus available fatty acids are entirely fluxed into PHA synthesis, resulting in higher PHA production (Kang et al. 2011). With an aim to produce mcl-PHA homopolymers and to control repeating monomers composition, inactivation of *fadB* and *fadJ* genes in the β-oxidation pathway from *E. coli* LS5218 has been performed (Tappel et al. 2012). The subsequent weakening in β-oxidation caused enoyl-CoA buildup, which later polymerized to PHAs by a (*R*)-specific enoyl-CoA hydratase (PhaJ4) and PHA synthase PhaC1(STQK) mutant. Without being degraded by β-oxidation, the fatty acids supplemented were directly turned to PHAs, having a number of repeating units similar to those of the fatty acid's carbon number. This enables controlling the composition of repeating units just by matching the corresponding fatty acids with similar carbon length for desired repeating unit (Table 5).

In fermentation studies, various methods are employed to obtain higher cellular production; using a pH-stat mode is one such method. In pH-stat mode feeding strategy, feeding of nutrients is accomplished when pH rises as a result of depletion of carbon source. Fed-batch fermentation strategy of *Pseudomonas oleovorans* using a pH-stat mode was performed for achieving high-density cell cultivation and resulted in high PHA accumulation by supplementation of octanoic acid as a substrate (Kim 2002). Co-feeding of octanoic acid with nitrogen at (C/N) ratio of 20 g octanoic acid per gram ammonium nitrate accumulated up to 75% of polymer content, which was considerably high. However, it was reported that at (C/N) of 10 g octanoic acid successfully attained the highest values for CDW (63 g/L) and productivity (1.0 g/L/h).

Alternatively, jatropha oil from *Jatropha curcas* can also be used instead of using food-grade oils and has considerable potential benefits for bioplastic industry. Jatropha is easy to plant, grows quickly, is very robust, and is highly tolerant toward drought, thus making it a cheap sustainable oil source. Jatropha plantations generate large amounts of non-edible oil, with the fatty acids composition mainly consisting of linoleic, oleic, palmitic, and stearic acid (Akbar et al. 2009). The potential of jatropha oil to serve as a feedstock for PHA production was first evaluated using a wild-type strain, *R. eutropha* H16. The resulting yield at 0.78 g PHA per g of jatropha oil produced from a mixture of jatropha oil at 12.5 g/L and urea at 0.54 g/L were comparable with that of other plant oils, as describe previously (Ng et al. 2010). P(3HB) produced from jatropha could also be accumulated as high as 87 wt.% polymer from 13.1 g/L of CDW. Similarly, urea has been reported as the preferable source of additional nitrogen for this cultivation, as it resulted in higher cell mass production and PHA accumulation. Although non-edible jatropha oil is toxic to humans and most vertebrates (Jing et al. 2005), the toxins did not affect the bacterial strain used in P(3HB) production. Subsequently, P(3HB-*co*-3HV) with 42 mol% 3HV was acquired using similar strain. Moreover, synthesis of P(3HB-*co*-3HHx) with 3 mol% 3HHx was demonstrated by *R. eutropha* PHB^{-}4/pBBREE32d13

Table 5 Biosynthesis of mcl-PHA polymers from fatty acids using various engineered strains of microorganisms

Strains	Fatty acid	PHAs	CDW (g/L)	Polymer content (wt. %)	Reference
Pseudomonas putida KTMQ01	Octanoate (C8)	P(3HHx-*co*-3HO-*co*-3HD-*co*-3HDD)	4.02	86	Cai et al. (2009)
	Decanoate (C10)	P(3HHx-*co*-3HO-*co*-3HD-*co*-3HDD)	1.69	27	
	Dodecanoate (C12)	P(3HHx-*co*-3HO-*co*-3HD-*co*-3HDD)	2.75	30	
Pseudomonas putida KT2442	Octanoate (C8)	P(3HHx-*co*-3HO-*co*-3HD)	4.64	46	Wang et al. (2011)
Pseudomonas mendocina CH50	Octanoate (C8)	P(3HO)	0.64	23	Rai et al. (2011)
Pseudomonas putida KTQQ18 recombinant	Decanoate (C10)	P(3HD)	1.43	11	Liu et al. (2011)
Pseudomonas entomophila LAC26 recombinant	Dodecanoate (C12)	P(3HDD)	2.70	91	Chung et al. (2011)
Escherichia coli CAG18496 recombinant	Decenoate (C10)	P(3HD)	0.54	27	Sato et al. (2012)
	Octenoate (C8)	P(3HO)	0.58	21	
Escherichia coli LS5218 recombinant	Dodecanoate (C12)	P(3HDD)	1.03	29	Tappel et al. (2012)
	Decanoate (C10)	P(3HD)	1.03	26	
	Octanoate (C8)	P(3HO)	0.86	47	

mutant strain, expressing the PHA synthase from *A. caviae* (Ng et al. 2011). The presence of a higher amount of HV precursor, sodium valerate, and sodium propionate along with jatropha oil increased the 3HV incorporation while reducing the CDW and PHA content. Moreover, as previously reported, at high (C/N) ratio of 30, maximum yield in biomass and greatest PHA accumulation were achieved. Both studies revealed that jatropha oil could assist in cell growth and biosynthesis of both homopolymeric and heteropolymeric PHAs by using the native and engineered strains of *R. eutropha*. The characteristics and properties of P(3HB-*co*-3HV) and P(3HB-*co*-3HHx) thus synthesized were also similar to those produced from other plant oils and sugars.

Thus, compared to sugars, plant oils provide a great advantage in respect of PHA yield, when considering the choice of feedstock for PHA production. Although some effective producers of PHAs have been reported, nonetheless, the average PHA yields are quite low and still insufficient for cater the needs. Therefore, more comprehensive work is needed to accelerate microbial growth and improve the fermentation technology utilizing plant oils as substrates in producing PHA.

4 Applications, Prospects, and Policies for Bioplastics

Recently, interest in bioplastics produced from renewable resources has increased significantly due to their sustainable benefits. Polyhydroxyalkanoates are natural biopolymers synthesized by bacteria upon utilizing resources from agricultural activities. These materials can be processed to produce useful range of products, and their biodegradability and eco-friendliness are beneficial, especially for disposable packaging and other sustainable applications. Today, bioplastics can be found in various segments, including food packaging and services, agriculture and horticulture, consumer electronics, automotive manufacturing, consumer goods, and household appliance as well as in medical and pharmaceutical applications.

Food packaging sector is the largest growth segment in the polymer processing industry, where the advancements in functionality such as compostability and sustainability are attracting greater consumer interest. Biodegradable food packaging has been successfully commercialized, with different bioplastics being used for various packaging solutions. Biodegradability is an important requirement for perishable food packaging products. Currently, most of the bio-based packaging stuff are in the form of films, trays, or containers that are used for packaging of short shelf life products such as ready-made foods, fresh fruits and vegetables, juices and beverages, as well as long shelf life products such as snacks, noodles, and dried foods, which do not require high oxygen and water vapor protection (Peelman et al. 2013). Other packaging options also being utilized are the easily disposable products including food utensils, cups, plates, cutleries, or carrier bags. These products are suitable for outdoor events, festivals, use in various modes of transports, or even at some cafeterias where disposable stuffs are frequently used. In order to cater the high demand, Metabolix Inc., USA, produced variants of biopolymers marketed under Mirel® brand, which are suitable for a wide range of food products and for various solutions such as frozen food storage, microwave reheating, as well as for hot water.

However, the major drawback currently being faced by the food packaging industry is to enhance the packaging durability along with the product shelf life. The concept of “active packaging” has been introduced lately, as an innovation idea that could provide positive interaction of the food with packaging materials. This can be achieved by addition of certain additives for improvement of the plastic performance by serving as an inert barrier to the external environment, while at the same time prolonging the food’s shelf life. Moreover, the incorporation of some

absorbers in the polymer materials may prevent the foods from deterioration caused by the undesired constituents such as oxygen, carbon dioxide, methane, and water vapor. Among the most interesting developments in active packaging are the introduction of antimicrobial (Quintavalla and Vicini 2002) and antioxidant species (Gómez-Estaca et al. 2014) as packaging components. The use of antimicrobial packaging released an active compound that reduces the microbial growth and allows the food to be preserved for longer time. Some nanoparticles and nanomaterials have also been clarified for their antimicrobial activity (Khosravi-Darani and Bucci 2015) and applied in antimicrobial packaging films. Similarly, antioxidant species that able to absorb oxygen and protect the foods from being oxidized may affect the food quality and changes in its nutritional and chemical composition.

Biodegradable polymers also offer exclusive advantages in agricultural and horticulture industries by minimizing the use of chemical fertilizer and promoting organic farming. Biodegradable plastics are mainly used as plant pots and growing bags as well as for controlled release of fertilizer. Planters can transfer potted plants directly into the ground, and later the plastic gets degraded naturally by the action of indigenous bacteria that are present in the soil. Degradation and composting process released essential material for the plants to grow, thus reducing the dependence upon chemical fertilizers. Moreover, by coating fertilizers (Philip et al. 2007) with encapsulated PHA resins, nutrients are protected from leaching and are released in controlled amount by slow degradation. This means that all the nutrients are fully consumed by the plant and will not be wasted due to leaching process. Considering that nutrient uptake is efficient and effective, this process will have a minimal impact on the environment.

PHAs are also utilized in the marketplace as packaging films predominantly used for food wrappings, paper coatings, and shopping bags. Prior to that, in 1990s, PHAs were firstly used as packaging material, in containers for shampoo bottles by Wella AG, Germany (Chen 2011). Later, several companies including Biomers, Metabolix, Polyone, Procter & Gamble Co., and a few others started using PHAs for diverse disposable items such as cosmetic containers, diapers, hygiene products packaging, razors, stationaries, utensils, and other household appliances.

In recent years back, PHAs have significant contribution in the treatment of diseases and improvement of healthcare. A lot of effort has been made on PHA application in the development of artificial organ, drug delivery, medical devices, surgical garments, tissue engineering, therapeutic uses, and wound dressings (Wu et al. 2009). A class of PHA family, mcl-PHAs, has a good prospect in biomedical implants such as tissue and organ regeneration scaffolds, e.g., arteries, vascular grafts, heart valves, and nerve cells. Meanwhile, high mechanical strength PHAs, mainly scl-PHA like P(4HB) and P(3HB-*co*-3HV), were also used for surgical sutures (Shishatskaya et al. 2004), hard tissue treatment like bone tissue repair (Galego et al. 2000), and regeneration of cartilage (Wang et al. 2008). Also, research for skin regeneration via PHA scaffolds has been carried by electrospinning PHA heteropolymers to fabricate nanostructured fibrous scaffolds utilizing P(3HB-*co*-4HB) and P(3HB-*co*-3HHx) (Ying et al. 2008).

In drug delivery system, PHA biopolymers are primarily used as subdermal implants, compression-coated tablets for oral administration, as well as particulate spheres for intramuscular, intraperitoneal, intravenous, and subcutaneous therapy (Nigmatullin et al. 2015). Drugs such as antibiotics (Türesin et al. 2001), anticancer agents (Masood et al. 2013), and vaccines (Shrivastav et al. 2013) can be entrapped or microencapsulated in PHA microstructure. They can be utilized for in vivo localized drug delivery, along with the controlled rate and gradual release of the therapeutic agent for a certain period of few days to few months. In pharmacology, degradation of PHA biopolymers in the target host offers a great extent of efficacy and effectiveness of the drug treatment by coupling this phenomenon with the controlled release of bioactive compounds. For instance, P(3HB-*co*-3HHx) was reported in the fabrication of anticancer agent-loaded nanoparticles for targeted cancer treatment (Kılıçay et al. 2011). Incorporated etoposide together with folic acid attached onto the P(3HB-*co*-3HHx) nanoparticles was observed to have high cytotoxic effects on HeLa cells. In targeted drug delivery (Yao et al. 2008) and regenerative medicine (Dong et al. 2010), PhaP, also known as phasin, a PHA granule binding protein which is amphiphilic in nature, has shown an ability for binding to the hydrophobic polymer core surface through strong hydrophobic interaction. Fusion of PhaP with arginyl-glycyl-aspartic acid, RGD, produced by recombinant *E. coli* was used for coating the hydrophobic surfaces of biopolyesters for promoting cell adhesion and proliferation (Dong et al. 2010; Li et al. 2015).

Automotive industry manufacturers have also shifted to bio-based reinforced plastics in the production of solid interior and exterior components with standard safety features. The products include seat, door panels, dashboard, airbag covers, steering wheels, and floor mats. Another emerging application of PHA has been observed in large proportion of consumer electrics and electronics appliances. A wide range of bioplastic products have been introduced into the electrical components such as casings and circuit board as well as for fast-moving consumer electronic products including mobile accessories, speakers, earphones, and computer casings. In the future, the use of bioplastics will not be restricted to daily consumer products and medical sectors only, but can be also be extended to highly advanced technology application. For example, new sophisticated applications of bioplastic can be realized in development of biosensors and compatible electromedical devices for healthcare.

In order for a product to be claimed and classified as "biodegradable," the product itself must fulfill all the requirements according to the standards set by European Norm EN 13432, the US ASTM D6400, or ISO 147088. All standards require that biodegradable products be completely degraded and decomposed in composting facilities within a specific time frame, without leaving any harmful effects or toxic residue. All standards state that the characteristics of degradable material must show the capability to convert into carbon dioxide by the action of microorganisms. However, degradation rate defined by each standard might be different, as stricter EN 13432 required 90% degradation within 180 days.

Recently, production process for bio-derived polyethylene (PE) and polyethylene terephthalate (PET) for plastic bottles has been developed. However, bio-derived PE

and PET had similar chemical structure to those derived from fossil oil. Therefore, it is difficult to distinguish between these two in order to be regarded as a green plastic and obtaining certification. A simple method to identify the differences between these two has been established by using an accelerator mass spectrometry. From the sample analysis, biomass content can be determined by measuring the amount of radioactive isotope ^{14}C, present in the sample. However, this current method is uneconomical (quoted at around 580 US dollars per sample), which is relatively quite expensive for regular inspection and identification purpose. It is hoped that in the near future, further technology advancement will enable the detection of biomass content in a plastic material efficiently, thus giving an extra advantage for placing bio-based product at higher position in the consumer market.

5 Conclusion

Research and development in the field of PHA production has been driven by the limited availability of fossil fuel resources, hiking of the petroleum price, and concerns over environmental issues. In the meantime, significant progress has been observed over the last few decades, with innovations of existing technologies and development of novel engineering approaches in the bioproduction of PHAs. The potential of various native PHA-producing bacterial and recombinant strains has been exploited further in order to increase PHAs' yield and productivity. However, the major limitation for extensive application of PHAs is associated with their high production cost, expensive raw materials, and complicated downstream processes. In this regard, work is underway, looking for reliable processes utilizing cheap raw material from agricultural activities, so that the production cost can be lowered, thus enabling PHAs to compete with the plastics produced from fossil oil. The remarkable production of PHAs utilizing oil fats demonstrates them as a promising resource with greater advantages leading toward commercialization. Moreover, one major progress in PHA improvement allows for a greater usage of these materials through the development of heteropolymers to overcome the limitations in properties that earlier restricted their use. This has increased the application of these biopolymers in variety of fields, most notable being the packaging and biomedical applications.

6 Opinion

Despite the existing challenges on increasing PHA yield and improving bacterial strain efficiencies, the ongoing cutting-edge research, along with the latest development in modern biotechnology, allows for predictive engineering for metabolic pathways, accelerated process efficiencies, and manipulating features for producing tailor-made PHAs that are suited for diverse usage. An integration of more

productive recombinant strains capable of consuming cost-effective carbon sources with efficient fermentation process will certainly pave the path toward higher production efficiency. Moreover, the versatility of plant oils has been demonstrated in the development of various PHAs leading to new applications. Despite all this work, there is still a lot of scope for improvement in the current technology. This suggests further process optimization and upscaling studies for validating large-scale production. These are required for ensuring the consistent product quality, maintaining optimum conditions, and, thus, minimizing microbial stress and enhancing metabolic efficiency. Development of antibiotic-free PHA production methods, the choice of operation strategy, and applicability of effective separation and purification would also significantly impact on PHAs throughput by lowering the production cost. With all these efforts, it is hoped that PHAs will lead the plastic market in the near future, thus helping to reduce the environmental problems associated with waste disposal. The ongoing commercialization projects are soon expected to make PHAs available in the market for various applications.

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Microbial Synthesis of Polyhydroxyalkanoates: Diversification

Qi Wang and Changshui Liu

Abstract White pollution and fossil oil shortage lead to a rising demand for alternatives to petrochemical-derived plastics. As biodegradable and biocompatible plastics, polyhydroxyalkanoates (PHAs) can be entirely synthesized by microorganisms via a series of enzymatic reactions from bio-renewable feedstocks. The bio-based PHA exhibits the similar physical properties to petroleum-based polymers. The PHA material properties strongly rely upon the number of carbon atoms in the main chain of the monomers, which divided PHA into two main categories, SCL PHA and MCL PHA. So far, all the types of PHA are biosynthesized via three pathways closely related to the intermediates of glycolysis, fatty acids metabolism, or synthesis. In efforts to develop new products, recombinant strains have been applied to produce various polymers. According to the ratio and arrangement of the individual monomer integrating into the chains, novel PHA are comprised of homopolymers and copolymers, the latter including random and block copolymers. They each possess distinctive but complementary competitive advantages in microstructure, thermal, and elastomeric properties.

Keywords Polyhydroxyalkanoate (PHA) • Biodegradable plastics • Microorganism • Enzymatic reaction • Biosynthesize • Homopolymers • Copolymers

1 Introduction

In recent years, bio-based sustainable plastics have attracted considerable attention due to the alarming rates of depletion of petroleum reserves and serious environmental problems derived from plastics pollution (Chen and Patel 2012; Somleva et al. 2013; Soroudi and Jakubowicz 2013). There are several kinds of bio-based

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plastics including starch-based polymers, poly(lactic acid), biopolyethylene (bio-PE), PHA etc., among which polyhydroxyalkanoate (PHA) polymers are recognized as the only one which can be not only completely synthesized but also degraded by microorganisms (Chen 2010; Keshavarz and Roy 2010; Krueger et al. 2015). PHA represents the biopolymers produced by various microorganisms to store carbon source and reduce equivalents usually in response to unfavorable growth conditions (Lenz and Marchessault 2005; Philip et al. 2007). The PHA history dates back to early last century when sudanophilic, liquid-like inclusions were firstly discovered in *Azotobacter chroococcum* (Madison and Huisman 1999; Sudesh et al. 2000). Later, poly(3-hydroxybutyrate) (PHB) was identified by chemical composition analysis (Steinbüchel and Lütke-Eversloh 2003). Since then, over 150 kinds of monomers with diverse structure have been identified (McChalicher and Srienc 2007; Keshavarz and Roy 2010). Such a rich pool of PHA monomers make it possible to constitute a wide variety of microbial polymers. Most recently, many plants have been found to allow the PHA synthesis (Wang et al. 2010a, b; Ariffin et al. 2011; Dalton et al. 2011).

No matter in native organisms or in recombinant ones, PHA polymers can be synthesized via enzymatic reactions to convert bio-renewable resources (Patel et al. 2012; Morgan-Sagastume et al. 2014; Kumar et al. 2015a, b). Thereby the kind of PHA polymers usually depends on several factors: metabolic ability of host microorganisms, substrate specificity of synthases, sorts of carbon source, and PHA synthesis pathway (Gao et al. 2011). Based on the number of C atoms in the main chain of the monomers, PHA polymers can be classified into two main categories (Fig. 1): short-chain-length (SCL) PHA and medium-chain-length (MCL) PHA. Monomers with 3–5 carbon atom units on aliphatic chain are in SCL classes, such as 3-hydroxypropionic acid (3HP), 3-hydroxybutyric acid (3HB), 4-hydroxybutyric acid (4HB), and 3-hydroxyvaleric acid (3HV), while monomers with 6–14 carbon atom units belong to MCL classes, such as 3-hydroxyhexanoic acid (3HHx), 3-hydroxyheptanoic acid (3HHp), and 3-hydroxytetradecanoic acid (3HTD) (Hazer and Steinbuchel 2007). Another classification method is based on the types and arrangement of the individual monomer incorporating into the polymer chains (Fig. 2). Based on this, three types consisting of homopolymer PHA, copolymer PHA, and block copolymer PHA are proposed (Gao et al. 2011).

The PHA physical properties strongly rely upon the structure and arrangement of the monomers (Feng et al. 2004). For instance, SCL PHA and MCL PHA generally have thermoplastic properties and elastomeric properties, respectively, while the copolymer of SCL PHA and MCL PHA usually combines properties of both types (Lu et al. 2009). The versatility of PHA monomer and diverse combination strategy enable PHA polymers to span a wide range of material properties, some of which exhibit resembled and competed material properties compared to petroleum-based polymers. Apart from the production of PHA polymers from bio-renewable feedstocks and their excellent physical properties, another special reason why the PHA polymers have been under extensive investigation is that they are thoroughly biodegradable (Krueger et al. 2015). Polymers can be fully converted and mineralized to CO_2 and water by PHA depolymerase secreted by soil microorganisms,

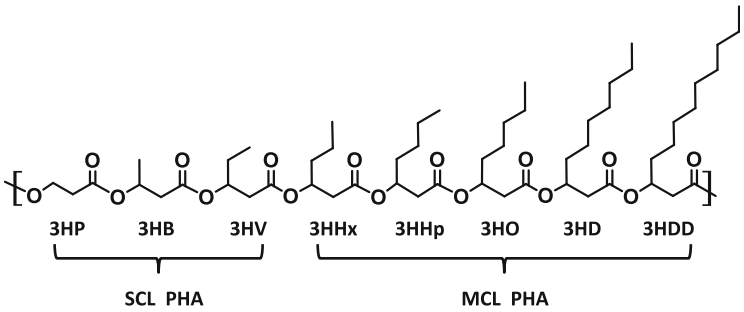

Fig. 1 Chemical structure of PHA monomers

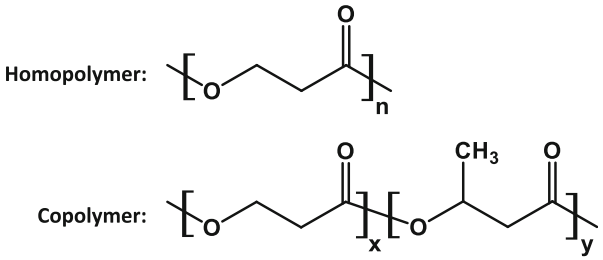

Fig. 2 Chemical structures of PHA homopolymer and copolymer

which effectively alleviate the environmental pollution issue (Sridewi et al. 2006; Chanprateep 2010; Rehm 2010).

Furthermore, PHA polymers are also biocompatible, insoluble in water and piezoelectric. These characteristics facilitate the application of PHA polymers into a wide array of areas ranging from common material industry to special medical industry (Chen 2009; Lu et al. 2009; Rehm 2010). In this context, PHA polymers have been regarded as promising alternatives to conventional petroleum-derived polymers (Philip et al. 2007). This chapter focuses on biosynthesis of different types of PHA polymers and their application in various areas.

2 Biosynthetic Pathways for PHA

Generally, there are three main pathways for the biosynthesis of PHA (Fig. 3) (Philip et al. 2007; Chen et al. 2015). Pathway I is a representative by *Ralstonia eutropha* (Anderson and Dawes 1990). In this pathway, the generation of 3-hydroxybutyric acid (3HB) monomer starts with the condensation of two molecules of acetyl-CoA derived from glycolysis pathway to form acetoacetyl-CoA molecule under the catalysis of β-ketothiolase. Afterwards, 3-hydroxybutyryl-CoA is formed from the acetoacetyl-CoA molecule reduced by acetoacetyl-CoA reductase to act as the monomer to further synthesis PHB (Philip et al. 2007). Pathway II

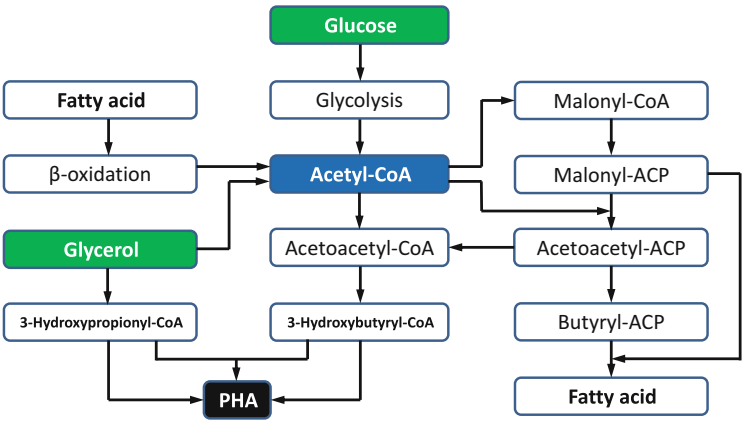

Fig. 3 Biosynthesis pathways for PHA

belongs to the rRNA homology group I of *Pseudomonas*. In this pathway, the intermediate product acyl-coenzyme A of fatty acid metabolism pathway (β-oxidation) are employed as precursors for PHA synthesis. MCL PHAs are mainly accumulated in *Pseudomonas* and the kind of PHA is highly related to the carbon substrate the strains feed on. Taking *Pseudomonas oleovorans* as an example, when alkane with even carbon atoms is used, only PHA monomer with even carbon atoms is synthesized, while when alkane with odd carbon atoms is employed, the result is opposite (Witholt and Kessler 1999). Pathway III is also found in *Pseudomonas* when fatty acid de novo biosynthesis is the main route. Except *P. oleovorans*, almost all the rRNA homology group I *Pseudomonas* can accumulate MCL PHA from carbon source like glucose, acetate, sucrose, or fructose (Anderson and Dawes 1990). As the intermediates of fatty acids biosynthesis are usually in the form of acyl-ACP-CoA, an enzyme named acyl-ACP-CoA transacylase is needed to convert it to the CoA form for the PHA synthesis (Rehm et al. 1998).

3 Biosynthesis of PHA Homopolymers

PHB is a wide representative polymer type in PHA family and was firstly discovered in *Bacillus megaterium* by Lemoigne in 1925 (Doi 1990). It was not realized that other bacteria may have the ability to accumulate PHA until Wallen and Rohwedder got polymers containing both 3HB and 3HV monomers isolated from activated sludge in 1974. Since then, more than 90 genera and 300 species of various bacterial strains have been reported to carry the ability to accumulate PHA granules, including archaebacterial, gram-positive and gram-negative bacteria, and photosynthetic bacteria, with nutrient limitation and carbon source excess (Suriyamongkol et al. 2007). PHB can be produced in several kinds of microorganisms, mainly in prokaryotes, especially bacteria such as *R. eutropha*,

Brevibacillus invocatus, *Halomonas* sp., and several strains of *Bacillus* and *Methylotrophs* (Thakor et al. 2005; Pandian et al. 2009; Chee et al. 2010; Sankhla et al. 2010; Jin et al. 2013; Kumar et al. 2013; Naranjo et al. 2013; Singh et al. 2013; Patel et al. 2015). There are also a few reports on production of PHA in eukaryotes, such as *Candida tropicalis* (Priji et al. 2013).

All of these microorganisms, *R. eutropha* serves as a model organism for exploration of PHB metabolism and has been under intensive investigation for more than 50 years (Reinecke and Steinbüchel 2008; Brigham et al. 2010; Wang et al. 2010a, b; Franz et al. 2011; Spoljaric et al. 2013). Furthermore, as the most common wild-type strain for the industrial production of PHB, *R. eutropha* could reach over 200 g/L cell density containing over 80% PHB in a 1 m^3 fermenter after 60 h of fermentation (Chen 2009). Mass production of PHB was carried out in a 60 L fermenter under the limitation of phosphate, resulting in 232 g/L PHB concentration and 281 g/L final cell concentration with a 3.14 g/L/h productivity (Ryu et al. 1997). *Alcaligenes latus* is another well-known wild PHB production strain and accumulate PHB in a growth-associated manner even under nutrient-sufficient conditions. A newly isolated strain named *Burkholderia* sp. USM was proved to produce PHB from triglycerides, fatty acids, and by-products glycerol, and up to 70 wt% of PHB could be obtained (Chee et al. 2010). Among the cheap carbon substrates, C_1 carbon sources have become the focus due to their significant function in greenhouse problem. There are already large amounts of research investigating PHB production using various C_1 carbon sources (Khosravi-Darani et al. 2013). *Methylobacterium organophilum* was reported to have a high yield of PHB using methanol as carbon source under potassium-limited condition (Kim et al. 1996). *Methylobacterium extorquens* also carries the ability to produce PHB from methanol. After the optimization of feed composition, the productivity of PHB reached to 0.98 g/L/h, which is higher than the previous study (Mokhtari-Hosseini et al. 2009). Recently, a bacterium having CO-resistant hydrogen-oxidizing abilities was isolated (*Ideonella* sp. O-1) and proved to accumulate PHB from CO_2 in autotrophic condition. This strain was tolerant to O_2 and CO concentration up to 30% (v/v) and 70% (v/v), respectively, which were higher than that of *Cupriavidus necator* and *A. latus* (Tanaka et al. 2011). When it comes to the PHB production in eukaryotes, *Saccharomyces cerevisiae* harboring the PHA synthesis genes from *Alcaligenes eutrophus* accumulated PHB up to 0.5% of the cell dry weight (CDW) (Leaf et al. 1996). Recently, Priji et al. explored the capability of PHB production of an isolated strain (BPU1) of *C. tropicalis* from the rumen of the Malabari goat (Priji et al. 2013).

The ability to accumulate PHA in wild-type strains derives from long-term evolution and development, so there are some limitations in PHA production, such as slow growing, hard to lyse for PHA extraction, and native machinery for polymer degradation. Genetic engineering have been serving as a powerful tool for PHA production since the entire set of gene involved in PHB synthesis was cloned from *R. eutropha* in 1988 (Lenz and Marchessault 2005). *Escherichia coli* has been regarded as an effective recombinant system for PHB synthesis owing to the deep understand of its genetic background, metabolic pathway, fast growth, and absence of PHA depolymerase system (Akaraonye et al. 2010). Recombinant *E. coli*

harboring PHA synthesis genes from *A. eutrophus* was able to produce PHB with 81.2 g/L concentrations and 80.1% CDW using a complex medium comprising a great deal of yeast extract and tryptone in fed-batch culture (Lee et al. 1994). By employing PHA synthesis genes from *A. latus*, another recombinant could accumulate PHB with 96.2 g/L concentrations after 37.5 h culture using a concentrated whey solution as carbon source (Ahn et al. 2000).

In contrast to PHB, there are only a few reports on other PHA homopolymers, such as poly(3-hydroxypropionate) (P3HP) (Andreessen et al. 2010), poly (4-hydroxybutyrate) (P4HB) (Steinbuchel et al. 1994; Zhou et al. 2012), poly (3-hydroxyvalerate) (PHV) (Shen et al. 2009b), poly(3-hydroxyhexanoate) (PHHx) (Jian et al. 2010), and poly(3-hydroxyheptanoate) (PHHp) (Wang et al. 2009). Recently, Chen's lab developed various PHA homopolymer synthesis platforms utilizing recombinant *Pseudomonas putida*. MCL PHA copolymer synthesis has been well studied in *P. putida* by fatty acid metabolism pathway (pathway II), but no homopolymer in wild-type strain. In their studies, a β-oxidation weakened mutant was constructed with the deletion of R-3-hydroxyacyl-ACP:CoA transacylase gene (*phaG*) and several related genes involved in β-oxidation, and the mutants enabled the production of PHHx, PHHp, and near homopolymer of poly(3-hydroxyoctanoate) (PHO). Based on this, when the PHA synthase was replaced by other kinds, the recombinant strains could also synthesize PHB, P4HB, and PHV (Qiu et al. 2005; Ouyang et al. 2007; Ma et al. 2009; Liu et al. 2011; Wang et al. 2011).

Among the PHA homopolymers, P3HP is a newly discovered polymer in recent years exhibiting excellent material properties (Andreessen and Steinbuchel 2010). So far, there has been no report about the P3HP synthesis in natural organisms. The first report about P3HP homopolymer synthesis in recombinant strain arose in 2010 (Andreessen et al. 2010). Andreessen engineered a recombinant *E. coli* harboring glycerol dehydratase gene from *Clostridium butyricum*, propionaldehyde dehydrogenase gene from *Salmonella typhimurium* LT2 and PHA synthase from *R. eutropha*. The strain finally accumulated 1.42 g/L P3HP through fermentation under fed-batch conditions. Later, this pathway was improved via replacing the glycerol dehydratase gene *dhaB12* from *C. butyricum* with the one *dhaB123* from *Klebsiella pneumonia*, and meanwhile, the reactivating factor *gdrAB* of *dhaB123* was also co-expressed. After a series of fermentation optimization, the yield of P3HP increased to 10.1 g/L P3HP (46.4% CDW) (Wang et al. 2013a). As the loss of plasmid usually caused the instability of the strain, to deal with this problem, Gao et al. constructed a genetically stable strain by applying plasmid addiction system and chromosomal gene integration (Gao et al. 2014). Consequently, the highest P3HP production of 25.7 g/L was obtained from inexpensive carbon source glycerol. Another P3HP synthesis pathway available to form P3HP from inexpensive carbon source is malonyl-CoA route (Andreessen et al. 2014). As acetyl-CoA is a common intermediate in central metabolism, no limitation exists on carbon source supplement. Wang et al. introduce the genes of malonyl-CoA reductase from *Chloroflexus aurantiacus*, propionyl-CoA synthetase and acetyl-CoA carboxylase from *E. coli*, and PHA synthase from *R. eutropha* into *E. coli* host (Wang et al. 2012). Using glucose as the sole carbon source, the P3HP was synthesized with a yield of 1.32 g/L (0.98% CDW). In addition, an attempt to assemble P3HP was

Table 1 Biosynthesis of different PHA homopolymers by microorganisms

PHA	Yield (g/L)	Mn (10^5 Da)	Mw/Mn	Thermal properties		Substrate[a]	Host[b]	Reference
				Tg (°C)	Tm (°C)			
P3HB	81.2	–	–	–	–	GLU	Ec	Lee et al. (1994)
	157.1	–	–	–	–	GLU	Ec	Wang and Lee (1997)
	96.2	–	–	–	–	LAC	Ec	Ahn et al. (2000)
P3HV	28.3	0.7	3.5	−15.8	103	GLU	Ah	Shen et al. (2009b)
P3HHp	2.7	2.5	1.8	−32.1	–	HP	Pp	Wang et al. (2009)
P4HB	2.1	3.3	1.2	−47	61	BDO	Ec	Meng et al. (2012)
	7.8	1.0	2.1	–	–	GLU	Ec	Meng et al. (2012)
	3.7	–	–	–	–	GLY, PA	Ec	Kämpf et al. (2014)
P3HP	2.6	1.1	1.5	−17.9	78.1	PDO	Ec	Meng et al. (2012)
	1.3	0.7	1.6	7	77	GLU	Ec	Wang et al. (2012)
	10.1	1.1	1.6	–	–	GLY	Ec	Wang et al. (2013a)
	25.7	–	–	–	–	GLY	Ec	Gao et al. (2014)

[a]GLU glucose, LAC lactose, HP heptanoic acid, BDO 1,4-butanediol, GLY glycerol, PA propionic acid, PDO 1,3-propanediol
[b]Ec *Escherichia coli*, Ah *Aeromonas hydrophila*, Pp *Pseudomonas putida*

made by Wang et al. by constructing a recombinant *E. coli* employing L-aspartate decarboxylase and 3-hydroxypropionyl-CoA synthase of *E. coli*, β-alanine-pyruvate transaminase of *P. putida*, and PHA synthase of *C. necator*. Under shake flask condition, the recombinant *E. coli* produced 0.5 g/L P3HP from inexpensive carbon source, without any addition of precursors and coenzymes (Wang et al. 2014). A summary of the biosynthesis of some typical PHA homopolymers is illustrated in Table 1.

4 Biosynthesis of PHA Random Copolymers

Though PHB has been receiving widespread attention in PHA family, it is hard to be processed due to its inherent high stiffness, brittleness, crystallinity, as well as melting temperature (Noda et al. 2005; Singh et al. 2015). So the poor thermal and

mechanical performance has a negative effect on the application of PHB. As properties of PHA highly rely on the molecular structure and comonomer composition, random integration of another monomer to PHB chain is expected to be a feasible way to remarkably enhance the properties of PHB. A large number of investigations have been performed on production of random copolymer containing 3HB monomer, such as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) (Yim et al. 1995; Ahankari et al. 2011; Kumar et al. 2014, 2015a, b, 2016), poly(3-hydroxypropionate-*co*-3-hydroxybutyrate) (P(3HP-*co*-3HB)) (Nakamura et al. 1991; Kang et al. 1993), poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P(3HB-*co*-4HB)) (Vigneswari et al. 2009; Li et al. 2010), and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(3HB-*co*-3HHx)) (Chen et al. 2001; Loo et al. 2005). All these copolymers demonstrated significant improved physical properties compared with PHB, some of them have already got industrial application.

P(3HP-*co*-3HB) has received more and more attention owing to the beneficial properties introduced by P3HP. 3HP is a rare compound in natural organisms. In the period before the pathway from cheap substrate was developed, studies on 3HP containing copolymer synthesis focused on addition of precursors such as 1,5-pentanediol, 1,7-heptanediol, and 3HP, which were structurally related to 3HP (Nakamura et al. 1991; Shimamura et al. 1994; Valentin et al. 2000). The precursors usually have the disadvantages of high cost, poor solubility in water, and toxicity to strains (Wang et al. 2013d). The report on P(3HP-*co*-3HB) firstly assemble from cheap substrate was described by Fukui et al. (2009). An PHB-producing strain *R. eutropha* was engineered with the expression of several exogenous enzymes including malonyl-CoA reductase and propionyl-CoA synthase of *C. aurantiacus*, wherein 0.77 g/L P(3HB-*co*-3HP) was accumulated with 0.2–2.1 mol% 3HP content using fructose as the sole carbon source. Wang et al. developed a novel system to biosynthesize P(3HP-*co*-3HB) from inexpensive carbon source with completely controllable ratio and arrangement (Wang et al. 2013c). In this system, the combination of two paratactic synthetic pathways was invented with respective regulatory mechanism: the system of pT_7-IPTG and pBAD-AraC guided the pathway genes to participate in the synthesis of 3HP-CoA and 3HB-CoA, respectively. By changing the expression level of corresponding genes, the range of 3HP fraction contained in P(3HP-*co*-3HB) could reach from about 12 mol% to 95 mol%. As expected, the composition of copolymers completely affected the thermal properties via differential scanning calorimetry analysis.

Besides random copolymer P(3HP-*co*-3HB), several investigations on other random copolymers with 3HP unit have also been exploited, such as poly(3-hydroxypropionate-*co*-4-hydroxybutyrate) (P(3HP-*co*-4HB)) and poly(3-hydroxypropionate-*co*-3-hydroxybutyrate-*co*-3-hydroxyoctanoate) (P(3HP-*co*-3HB-*co*-3HH-*co*-3HO)) (Green et al. 2002; Qiu et al. 2004; Meng et al. 2012). A summary of the biosynthesis of some typical PHA random copolymers is given in Table 2.

Table 2 Biosynthesis of different PHA random copolymers by microorganisms

PHA P(A-*co*-B)	Monomer (mol %)		Yield (g/L)	Mn (10^5 Da)	Mw/Mn	Thermal properties		Substrate[a]	Host[b]	Reference
	A	B				Tg (°C)	Tm (°C)			
P(3HP-*co*-3HB)	10–26	74–90	0.6–1.7	2.0–3.7	2.1–2.9	−8.1–1.0	85–157	SUC, 3HP	Al	Hiramitsu et al. (1993)
P(3HP-*co*-3HB)	7–29	71–93	0.5–2.1	2.8–3.5	2.1–2.2	−2–3	143–166	SUC, 3HP	Al	Shimamura et al. (1994)
	25–88	12–75	0.1–1.2	1.1–3.0	2.2–3.3	−19–5	44–159	3HP, 3HB		
P(3HB-*co*-3HV)	98.4–67	1.6–33	0.3–4.1	–	–	–	–	GLU, PA	Ec	Yim et al. (1995)
P(3HP-*co*-3HB)	1.6	98.4	1.2	2.9	4.3	−1.9	162.3, 167.8	FRU	Cn	Fukui et al. (2009)
P(3HB-*co*-4HB)	82	18	6.1	–	–	−9.2	127.9	GLU, KG	Ec	Li et al. (2010)
P(3HB-*co*-4HB)	80.9	11.1	6.2	–	–	−4.4	131.5	GLU	Ec	
P(3HP-*co*-4HB)	16.6–88.5	11.5–83.4	0.0–6.2	2.0–2.4	1.28–1.32	−41.9–24.4	35.7–64.8	PDO, BDO	Ec	Meng et al. (2012)
P(3HP-*co*-3HB)	11.5–94.6	5.4–88.5	1.1–2.6	1.3–3.4	1.9–3.0	−22–3	68–168	GLY	Ec	Wang et al. (2013c)

[a]SUC sucrose, 3HP 3-hydroxypropionate, VA valerate, MEOH methanol, PEOH pentanol, HP heptanoate, 4HB 4-hydroxybutyrate, BDO 1,4-butanediol, BO γ-butyrolactone, 3HB 3-hydroxybutyrate, GLU glucose, PA propionate, FRU fructose, KG a-ketoglutarate, PDO 1,3-propanediol, GLY glycerol
[b]Al *Alcaligenes latus*, Ms *Methylobacterium* sp., Ec *Escherichia coli*, Cn *Cupriavidus necator*

5 Biosynthesis of PHA Block Copolymers

Though the physical properties of PHA random copolymers have obtained remarkable enhancement, the detrimental aging effects is still a problem. Microbial block copolymer provided a new approach to obtain PHA polymers with different structures and properties, because this kind of copolymer captured properties of every integrated block and led to additional properties that were not existing in random copolymer or polymer blending. Also, block copolymer systems exhibit aging resistance performance (Dai and Li 2008; Hu et al. 2011; Li et al. 2011; McChalicher and Srienc 2007; Shen et al. 2009a). The block copolymer has three structure forms: AB diblock, ABC triblock, and $(AB)_n$ repeating multiblocks (Bates 1991). So far, several kinds of block copolymer have been successfully synthesized including P3HB-*b*-P3HV, P3HB-*b*-P4HB, P3HP-*b*-P4HB, P3HB-*b*-P3HHx, P3HB-*b*-P3HP, and P3HHx-*b*-P(3HD-*co*-3HDD) (Pederson et al. 2006; Hu et al. 2011; Tripathi et al. 2012, 2013a, b; Wang et al. 2013b; Li et al. 2014) (Table 3).

The essential point of producing block copolymer is the sequential integration of monomers into an elongating alkyl carbon chain. Unfortunately, most investigations on block copolymer biosynthesis are concentrated in successive addition of different precursors, exposing microorganisms to various growth conditions orderly. The ratio and composition of the monomer in the block copolymer are totally controlled by the precursors. Nevertheless, addition of precursors has seriously hampered these polymers from large-scale production. Wang et al. explored a novel approach to synthetize P3HB-*b*-P3HP from low-cost carbon source (Wang et al. 2013b). A previous constructed recombinant *E. coli* was employed carrying two paratactic synthetic pathways with independent regulatory mechanism and formed the monomers 3HB and 3HP from two substrates, respectively. Controlling expression levels of the two pathway genes led to synthesis of P3HB-*b*-P3HP with various 3HP monomer contents from about 7 mol% to 75 mol%. Novel molecular structure and thermal properties are found in the block copolymers, which are unavailable in the blend of P3HB and P3HP homopolymer or P(3HP-*co*-3HB) random copolymers. A summary of the biosynthesis of some typical PHA block copolymers is given in Table 3.

6 Perspective

Microbial synthesis of PHA has drawn more and more attention owing to the increasing oil cost, environmental conservation, and its own biodegradability. A promising era for PHA material industry will certainly come and bring many opportunities and challenge. To meet the rising requirements for industry and our life, the production of PHA has to be developed based on low-cost and diverse properties. The cost must be advantageous compared with petroleum-based materials and controllable from substrates and fermentation processes. PHAs have

Table 3 Biosynthesis of different PHA block copolymers by microorganisms

PHA PA-*b*-PB	Monomer (mol %)		Yield (g/L)	Mn (10^5 Da)	Mw/Mn	Thermal properties		Substrate[a]	Host[b]	Reference
	A	B				Tg (°C)	Tm (°C)			
P3HB-*b*-P(3HV-*co*-3HHp)	72	12.7 +15.8	–	0.5	8.7	−23.6, 3.5	170.6	BA, HP	Pp	Li et al. (2011)
P3HB-*b*-P4HB	20	80	2.7	0.5	3.1	−47.3	54.2, 161.4	BA, BO	Pp	Hu et al. (2011)
P3HHx-*b*-P(3HD-*co*-3HDD)	48.9	15.8 +35.3	2.0	0.8	1.9	−43.1	33.5, 66.1	HX, DD	Pp	Tripathi et al. (2013a)
P3HP-*b*-P4HB	71	29	1.7	2.2	2.5	−20.2, −46.2	55.0, 67.6	PDO, BDO	Ec	Tripathi et al. (2013b)
	63	37	1.1	2.1	2.6	−21.6, −45.2	53.4, 66.8			
P3HP-*b*-P3HB	75	25	0.8	2.8	3.2	−20, −1	73, 164	FRU, GLY	Ec	Wang et al. (2013b)
	7.4–75	25–92.6	0.7–1.6	–	–	–	–			

[a]BA butyrate, HP heptanoate, BO γ-butyrolactone, HX hexanoate, DD dodecanoic acid, PDO 1,3-propanediol, BDO 1,4-butanediol, FRU fructose, GLY glycerol

[b]Pp *Pseudomonas putida*, Ec *Escherichia coli*

managed to be produced by genetic engineering strains from conversion of inexpensive carbon sources like glucose and glycerol, which has gathered many wisdom and innovation. Besides, various PHA polymers including homopolymers, random copolymers, and block copolymers now have been biosynthesized continuously through screening of strains, enhancement of processes, and utilization of new technology to overcome the bottleneck of high cost. Furthermore, novel PHA properties have been exploited in thermal stability, pressure resistance, plasticity, biocompatibility, etc. These new specialties will be advanced in areas of low-cost and high-value applications such as daily chemical packaging and biomedical materials. In the future, the popularization of the bio-based polymers will undoubtedly make great contribution to the sustainable development of economy, society, and environment.

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Microbe-Derived Itaconic Acid: Novel Route to Biopolyamides

Mohammad Asif Ali and Tatsuo Kaneko

Abstract Itaconic acid is a white crystalline organic compound with the formula $C_5O_4H_4$ and is classified as an unsaturated carbonic acid. Today, this organic acid is widely used for the production of resins, biofuel components, pharmaceutical products, artificial glass, plastics and in the textile industry and agriculture. Because many potentially useful substances can be prepared from this organic acid it can be considered a platform chemical. Using biotechnology, itaconic acid can be synthesized from *Aspergillus* sp. such as *A. itaconicus* and *A. terreus*, which have ability to produce the unsaturated itaconic acid. Itaconic acid has been particularly used in the vinyl polymer industries but recently we have used it for the development of novel biobased polyamides. Polyamides are industrially established compounds containing a –CONH– linkage, which can enhance the performance of materials, especially thermal and mechanical moduli. Itaconic acid-based polyamides contain a pyrrolidone ring, which constitutes the polyamide backbone. This pyrrolidone ring backbone is promising for the future development of biobased materials for a wide range of engineering plastics.

Keywords *Aspergillus citrus* • Polyamides • Pyrollidones • Polycondensation • Salt monomers

1 Introduction

Itaconic acid (dicarbonic acid) has been listed as one of the most capable and flexible future building blocks for the production of plastics, resins, and pharmaceutical products, and in agriculture and other industrial processes (Huang et al. 2014). The production of itaconic acid is from *Aspergillus terreus* and *Aspergillus itaconicus* using glucose as energy source. *Aspergillus* is one of the most powerful host organisms used in biotechnological pathways for the production of this promising substance (Corma et al. 2007), which can provide a wide spectrum of

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commercial products. The massive growing demand for fossil resources threatens future energy supplies so there is need to reduce demand by synthesizing biobased products (Steiger et al. 2013). Sustainable development is the key to reducing the use of petrochemical-based chemicals and changing to natural products. Global use of itaconic acid as a monomer for polymer manufacture and its production from biosynthetic pathways using carbohydrates via biological conversion can contribute to a biobased economy. The US Department of Energy includes itaconic acid in the list of top 12 platform chemicals. Biobased polymers have a great advantage over conventional polymers because they can contribute to a reduction in dependency on fossil-based polymers. Itaconic acid as a renewable organic acid, it contains two carboxylic groups and an unsaturated double bond (Corma et al. 2007). An unsaturated vinylidene group attached to carboxylic acid can be used as a vinyl monomer or comonomer for addition polymerization (Steiger et al. 2013), providing a direct alternative to substitution of acrylic acid or methacrylic acid (Isikgora and Becer 2015).

Polyamides (Nylon™) contain repeating units connected by –CO–NH– units and are widely used as high performance plastics in automobiles, medical equipment, and electronics as well as in construction (Guerra and Lima 2013; Ali and Kaneko 2015). However, environmental problems have recently attracted the attention of researchers to the development of biobased polyamides (Huang et al. 2014). Waste disposal is an urgent environmental problem because it directly affects the environment and can favor the enhancement of global warming (Steiger et al. 2013). Therefore, there is great need for the development of biobased plastics with controlled degradability for the establishment of a green and sustainable society. Many polymers only denature or degrade with difficulty (Guerra and Lima 2013). Biobased approaches lead toward a green and sustainable society (Hamer 2004). A wide variety of biobased nylons such as nylon-11, nylon-10,12, nylon-10,10, nylon-6,10, and nylon-6,12 (Baumann 1969; Ali and Kaneko 2015) have been commercialized. However, these polyamides have the limitation of low heat resistance as they contain a flexible chain (Aharoni 1997). Taking this into account, researchers are trying to improve polyamide performance. A rigid-ring structure is ideal for improving thermal and mechanical properties, while maintaining degradability (Ali and Kaneko 2015).

In this chapter, we discuss biobased polymers obtained from itaconic acid and copolymers identically produced using aromatic diamines, aliphatic diamines, and some different diacids as comonomers (Corma et al. 2007). Materials composed of these copolyamides demonstrate improved thermal and mechanical properties and also show degradable behavior in soil and photosolubilization under UV light (Ali et al. 2013; Ali and Kaneko 2015). Itaconic acid-derived N-substituted polypyrrolidones (polyamides) provide an environmentally friendly substitute for conventional plastics (Ali and Kaneko 2015) because itaconic-based polyamides exhibit higher performances.

2 Outlook for Itaconic Acid

Itaconic acid ($C_5H_6O_4$), a naturally occurring organic acid (Huang et al. 2014) produced from glucose-based media by *A. terreus* (Corma et al. 2007), is an unsaturated dicarboxylic acid with two pK_a values, a melting point of 167–168 °C, and density of 1.632 g/L at room temperature. Itaconic acid, also known as methylenesuccinic acid, is a five-carbon dicarboxylic acid and has the potential to be a key building block for polymer production. Its functional groups make itaconic acid effective for various reactions such as addition polymerization, production of anhydride, and as a monomer for formation of plastics, fibers, and elastomers (Steiger et al. 2013). Itaconic acid is used worldwide in the field of polymers and recently developed N-substituted pyrrolidone-based polyamides (Huang et al. 2014). The industrial production of itaconic acid is from carbohydrates via filamentous fungal fermentation using *A. terreus*, as shown in Fig. 1. Itaconic acid production was first observed in *A. itaconicus* in 1932 and current industrial fermentation production utilizes a related strain, *A. terreus*. It has been demonstrated that itaconic acid production in *A. terreus* follows glycolysis and the tricarboxylic acid (TCA) cycle (Corma et al. 2007). In these pathways, itaconic acid follows the TCA cycle as an intermediate and forms itaconic acid after decarboxylation of *cis*-aconitic acid. Initially, synthesis of itaconic acid proved to be uneconomic because of the high cost, but recently many researchers have overcome this problem. Because of the increased demand for itaconic acid, currently about 10,000–15,000 tons per year, many researcher are focused on ways of increasing production (Corma et al. 2007).

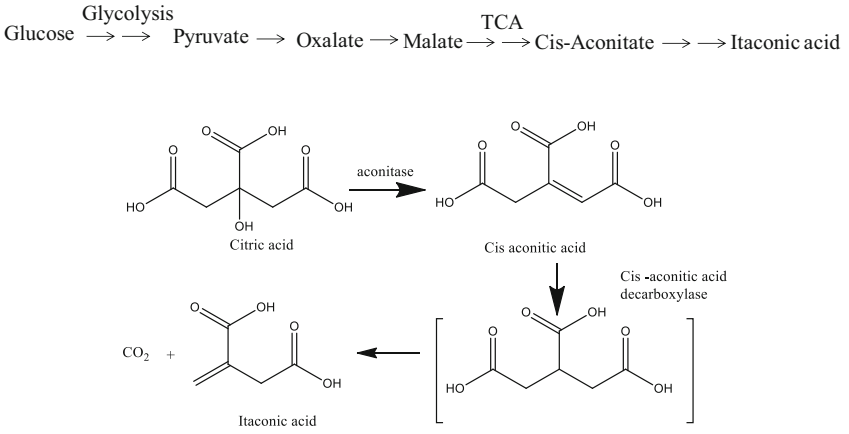

Fig. 1 Synthesis of itaconic acid from citric acid

3 Synthesis of Itaconic Acid-Derived Polyamides

Itaconic acid-derived polyamides are generally synthesized through a two-step process involving formation of a pyrrolidone ring backbone (Ali et al. 2013; Ali and Kaneko 2015). Previously, they were synthesized through direct interfacial mixing of monomers, which results in polymers with a three-way branched structure. Three-way branching, resulting from reaction of the unsaturated double bond with a terminal carboxylic acid group, restricts the formation of high molecular weight polymer. In a patent describing the mixing of itaconic acid and hexamethylene diamine to obtain low molecular weight polyamides ($M_w < 12{,}000$), formation of a pyrrolidone ring was confirmed, together with unusual three-way branching. During the condensation of monomer (itaconic acid and diamine) at high temperatures, the diamine easily vaporizes so it is difficult to maintain an equimolar monomer ratio (Ali et al. 2014). Different routes have been described that favor formation of high molecular weight polymer ($M_w > 30{,}000$). In the two-step method, nylon salt is first prepared and then used for melt polycondensation (Filgueiras et al. 2014). To avoid collapsing the 1:1 balance and to overcome the problem of low molecular weight, we selected the nylon salt method (Ali and Kaneko 2015).

To avoid side reactions, especially three-way branching, the nylon salt method is preferable for production of high molecular weight polymers. The process involves melt state polycondensation so it can proceed easily from laboratory scale to industrial process (i.e., bulk polycondensation). The reaction temperature is designed to be 5–10 °C above the melting temperature of the nylon salt (Filgueiras et al. 2014). Inside the polymerization flask, the first step is Michael addition with the loss of two molecules of water to form the stable five-membered ring (pyrrolidone ring) (Ali and Kaneko 2015). The amine reacts with the unsaturated double bond of itaconic acid, adding a further proton to the nearby carbonyl group; imino groups are formed, which instantaneously react with carbonyl groups to form the pyrrolidone ring (Fig. 2). The unsaturated double bond of carboxylic acid is more reactive toward the amine and can smoothly proceed to the condensation of

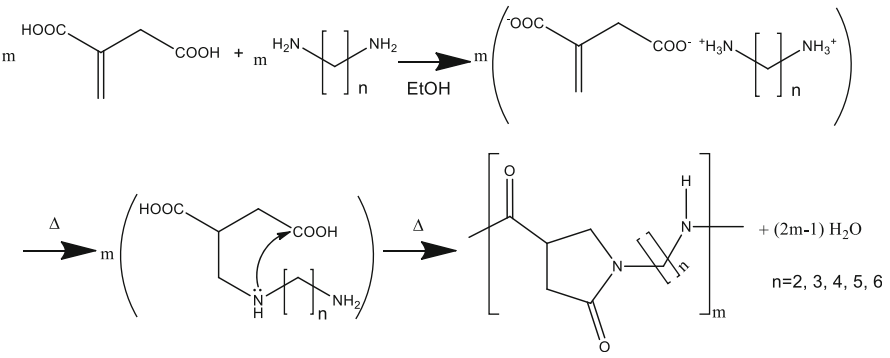

Fig. 2 Synthesis of biobased N-substituted pyrrolidone ring-based polyamides from itaconic acid salts with aliphatic diamine

water through cyclization, which means that Michael addition proceeds ideally (Ali et al. 2014).

4 Performance and Degradability of Itaconic Acid Polymers

Polymer performance depends on thermal stability, chemical resistivity, mechanical stability, and degradability. Itaconic acid-based polyamides have excellent thermal and mechanical stability. The pyrrolidone ring gives rigidity to aliphatic chains, but in aromatic-based polyamides it gives the extra benefit of thermal stability as a result of the thermally stable rigid aromatic ring. Aliphatic diamine-based polyamides show glass transition temperatures (T_g) and 10% weight loss (T_{d10}) values ranging between 80–152 °C and 300–410 °C, respectively (Ali et al. 2013, 2014; Ali and Kaneko 2015). In the case of aromatic polyamides, T_g and T_{d10} values are 156–240 °C and 370–400 °C, respectively. Polyamides based on heterocyclic rings, especially with *para* substituents, show higher stability and higher T_g (Ali et al. 2014). Generally, synthesized polyamides have different glass transitions; some T_g values are 199 °C (PA-a), 242 °C (PA-b), 156 °C (PA-c), and 173 °C (PA-d) (Fig. 3). Higher thermal stability provides sufficient heat resistance, meaning that these materials can be used as engineering plastics (Kaneko et al. 2006; Garcia et al. 2010; Banerjee and Maji 2011; Ahmed 2011). The intramolecular hydrogen bonding between the amide linkage with the rigid ring giving extra

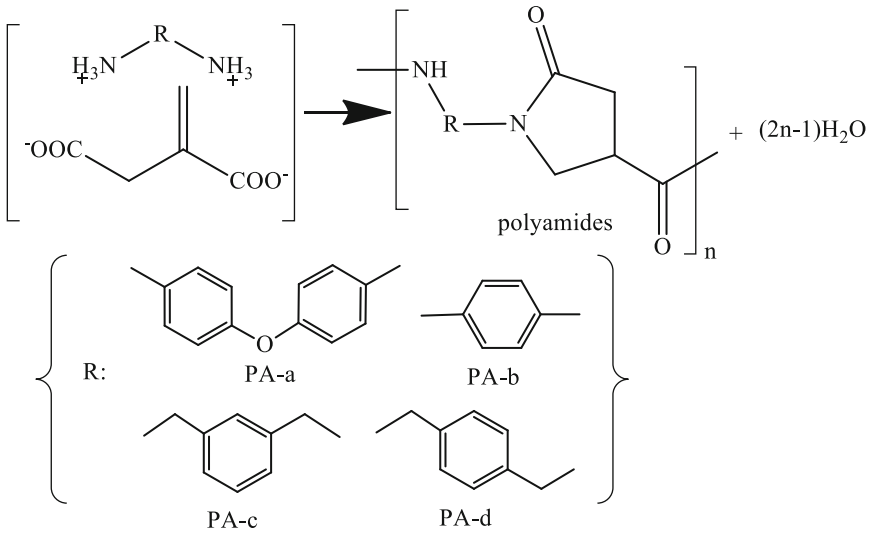

Fig. 3 Synthesis of biobased N-substituted pyrrolidone ring-based polyamides from itaconic acid salts with aromatic diamine

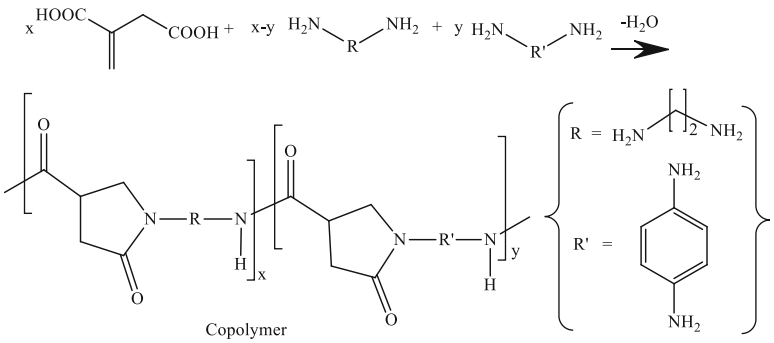

Fig. 4 Syntheses of itaconic acid-derived copolymer of phenylenediamine and ethylene diamine with itaconic acid

stability (Ali and Kaneko 2015). The T_g values achieved are higher than those for conventional nylons (57 °C for PA-66 and 53 °C for PA-6) but, on the other hand, T_{d10} was almost equal to values for conventional polyamides (390 °C for PA-66 and 400 °C for PA-6). The rigid pyrrolidone ring provides extra stability so the material can be stretched to obtain a fiber; however, the rigid ring lowers the breaking strength. The obtained tensile strength of aliphatic polyamide fiber was in the range of 65–165 MPa and Young's modulus was in the range of 430–2800 MPa. The rigidity of the N-substituted pyrrolidone ring in the polymer backbone reduces strain values at mechanical failure in the range 0.018–0.049; the polymer specimen showed less elongation (Ali et al. 2013, 2014). However, the extra rigidity of aromatic polyamides and copolyamides means that it is very difficult to prepare copolymers by introducing a flexible aliphatic chain together with the rigid ring while maintaining a high heat resistance. The presence of an aromatic ring together with the pyrrolidone ring reduces the tensile strength (65–68 MPa) and Young's modulus (430–1400), respectively (Fig. 4). Conventional polyamides with extensive amidic linkages (NH–OC bonds) absorb moisture as a result of intermolecular hydrogen bonding; in itaconic based-polyamides with NH–OC and N–OC bonds, the moisture absorption might be masked (Ali et al. 2013; Ali and Kaneko 2015). Moisture absorption by polyamides affects the structure–properties relationship, causing dimensional instability, reduced T_g, and inferior mechanical properties.

5 Syntheses of Aromatic-Aliphatic Copolymers

The rigidity of aromatic polyamides makes it very difficult to cast them as fibers (Garcia et al. 2010; Banerjee and Maji 2011; Ahmed 2011; Deopura and Padaki 2014). By incorporating a flexible aliphatic chain, we could improve fiber

formation. Incorporation of an aliphatic chain into the aromatic rigid backbone to maintain the thermochemical properties (Banerjee and Maji 2011) influences copolyamide performance because the aliphatic chain basically enhances flexibility (Ali et al. 2014). Flexibilty is enhanced by incorporation of an aliphatic chain, but the aromatic chain provides rigidity. We prepared copolyamides with different amounts of aromatic and aliphatic diamines using a fixed amount of itaconic acid. We then tested the performance of the prepared copolyamides containing different concentrations of aromatic and aliphatic diamines (Ali et al. 2013; Ali and Kaneko 2015). Different concentrations of diamine cause differences in segmental mobility and density, which affect the performance of materials (Ali et al. 2013; Ali and Kaneko 2015). Various copolymers were prepared by varying the aromatic/aliphatic diamine and concentration of itaconic acid (a 1:1 molar ratio of diamine: diacid is used for preparation of nylon salt) (Filgueiras et al. 2014). Small amounts of aromatic chain affect the structure–properties relationship of the resulting copolymer and can be used to modify the thermal properties and desired rheological properties (Ali et al. 2014). The prepared nylon salt was heated from 180 °C to 210 °C for 12 h. The prepared copolymer showed T_g values in the range of 119–148 °C and T_{d10} of 375–401 °C. After incorporation of an aliphatic chain inside the aromatic backbone, the tensile strength and Young's modulus of the resulting copolymers were extensively improved, 0.2–2.3 GPa and 20–70 MPa, respectively. The strain value of mechanical failure ranged between 0.036 and0.065, meaning that a pyrrolidone ring together with an aromatic rigid ring restricts higher elongation (Ali and Kaneko 2015).

6 Degradability of Biobased Polyamides

Degradability is a big issue in the polymer world, because polymer disposal can cause pollution and put pressure on the environment. Polyamides are poorly degraded in the natural environment and are considered resistant to biodegradation because of good chemical resistance, toughness, and excellent thermomechanical properties (Kaneko et al. 2006; Lendlein and Sisson 2011; Tolinski 2011; Iwata 2015). These properties make polyamides resistant to degradation in the natural environment, because the amide linkage (−NHCO–) provides strong interchain interactions (Osswald and Garcia-Rodriguez 2011; Pilla 2011). Some microorganisms, especially bacteria and fungi, can partially degrade conventional nylons such as nylon-6 and nylon-66. Nylon-6 degrades to oligomers through the action of *Flavobacterium* and *Pseudomonas* species but nylon-66 degrades through oxidation processes mediated by white rot fungal strains (Tolinski 2011; Pilla 2011; Dai et al. 2015). *Pseudomonas* species can completely degrade PA-4, although the polyamide degradation process is still undefined. Furthermore, from the viewpoint of carbon neutrality, in a biobased polymer the polyamide absorbs moisture and thus reduces polymer performance. However, N-substituted pyrrolidone ring-based

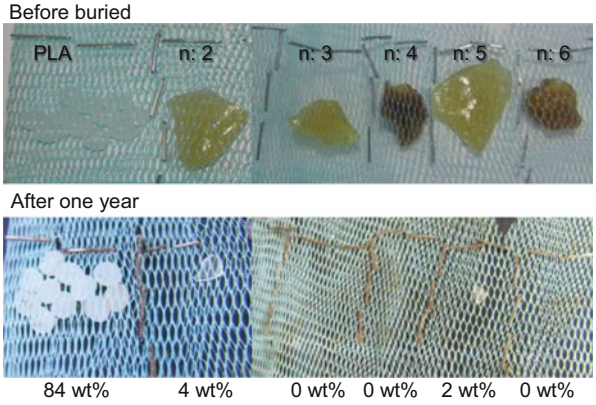

Fig. 5 Corrosive behavior of polyamides under soil

polyamides have an important advantage in that they absorb less moisture, only 4 wt% as compared with 11 wt% for conventional polyamide-9 (PA-6, PA-66). These N-substituted pyrrolidone ring-based polyamides can be dispersed in alkaline solution (pH 10) after vigorous stirring at 60 °C. Structure analysis using NMR has shown that ring opening is possible during dispersion in alkaline solution. Therefore, polymers were buried at a depth of 2–4 cm in soil of pH 7.5–7.9 and the changes in shape and weight examined after one year (Ali et al. 2013, 2014; Ali and Kaneko 2015). The N-substituted pyrrolidone ring-based polyamides synthesized from different diamines (1,3-diaminopropane, 1,4-diaminobutane, and 1,6-hexanediamine) had totally disappeared. However, some of the N-substituted pyrrolidone ring polyamides, especially those derived from cadaverine and ethylenediamine, showed weight loss values of 96 and 98 wt%, respectively (Fig. 5) (Ali et al. 2013); poly(lactic acid) was used as a reference. Aromatic polyamides showed degradation in soil, compared with poly(lactic acid). Polyamides derived from itaconic acid and diamines such as 4,4-diaminodiphenylether, *m*-xylenediamine, *p*-xylenediamine, and phenylenediamine showed weight loss values of 34, 100, 76, and 23 wt%, respectively (Ali et al. 2013, 2014; Ali and Kaneko 2015). The shape and weight loss of some of the most famous biodegradable and biocompatible thermoplastics (Kabasci and Stevens 2014), which were co-buried with the itaconic-based polyamide, showed only 16 wt%. The changes in polymer shape, weight, and color indicate the degree of corrosion in soil, which is affected by several physicochemical and biological processes. The water solubilization test was checked underwater using a high-pressure mercury lamp, the wavelength of which was similar to that of sunlight (280–400 nm). Hydrolysis of the pyrrolidone ring generates hydrophilic carboxyl side chains. Long periods of exposure to sunlight (which includes UV-A and UV-B) causes a gradual ring-opening reaction, overcoming the problem of waste disposal (Ali et al. 2013; Ali and Kaneko 2015). As a result, the polymer exhibits water solubility behavior (Fig. 6). The above results prove that if polyamide is exposed to sunlight under

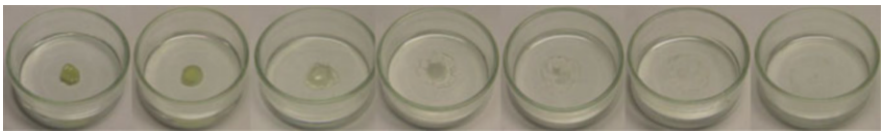

Fig. 6 Time-dependent water solubilization behavior of polyamide resins under UV-light

the water or left in landfill for long time, the shape, size and color of polymer are affected as a result of ring opening after hydrophilization (Ali et al. 2013; Ali and Kaneko 2015). Thus, biobased N-substituted pyrrolidone ring-based polyamides show higher performance and better environmental degradation than petroleum-based PA-66 (Ali et al. 2013, 2014).

7 Conclusions

Itaconic acid is a versatile diacid that is used as a monomer in the polymer industry. Currently, itaconic acid is mainly produced by fermentation of *A. terrus*. Because of the presence of an unsaturated double bond it has high potential for use as an additive in polymerization. The diversified nature of itaconic acid and the possibility of addition polymerization with the double bond have resulted in a reduction in the production of petroleum-based products, especially acrylic acid. Itaconic acid is used in development of environmentally degradable bioplastics derived using the heterocyclic ring in salts of itaconic acid and diamine. This new molecular design of biopolymers containing heterocyclic rings promises high performance materials and a solution to the problem of plastic waste. The water-solution method of biopolyamide degradation could help solve some serious problems for marine creatures (e.g., fishing lines left in the ocean). In spite of this advantage, there are still some drawbacks to biobased plastics that prevent their wider commercialization. However, itaconic acid and its polyamide are cheaper than similar conventional biobased polyamides (e.g., polyamide-11), which could have a large impact over a wide range of commercialization. The new biobased polyamides are expected to have a greater impact than conventional polyamides and significantly reduce the environmental strain and stress caused by the petroleum products. Bio-nylon has outstanding thermomechanical properties and degradability and can be used in applications ranging from commodity to hi-tech.

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Basics of Methanogenesis in Anaerobic Digester

Vinay Patel, Soumya Pandit, and Kuppam Chandrasekhar

Abstract The field of wastewater management and alternative energy are one of the most unexplored fields of environmental biotechnology. The biomethane is considered as renewable natural gas which can be derived from organic waste and sewage treatment. In recent past biomethane is emerging as a promising gaseous fuel utilized in a cogeneration or trigeneration power plant. Biomethane usually produced through anaerobic digestion in oxygen-deficient environment where a series of microorganisms convert the complex waste to biogas via liquefaction through a cascade of enzymes. Biomethane produced in anaerobic digester can be utilized for decentralized power generation, and additionally revenue can be gained in the form of a CO_2 credits and/or other greenhouse gas emission credits. In the present book chapter, the technical know-how of anaerobic digestion and biocatalyst associated with digester has been depicted. A thorough understanding of the fundamental principles of anaerobic digestion would help to perceive new aspects of bioenergy conversions. The book chapter highlights the concise of biochemistry for biomethane production as well as important major factors involved in the process toward the realization of a stable biomethane-based economy. Successful application of anaerobic digester was found in pilot-scale potential wastewater treatment along with renewable energy production. The proper configuration anaerobic digester and efficiently pretreated "feedstock" are key to maximize the production of methane. Therefore, basics of reactor design designs based on process economy have been discussed.

Keywords Anaerobic digestion • Methanogenesis • Anaerobic Reactor design • Wastewater management • Renewable energy generation • Acetogens • Acidogens • Methogens

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1 Introduction

The anaerobic digestion is a sustainable process which is gaining popularity in the realm of increasing demand of constant energy sources and concern of global warming due to emission of greenhouse gases. The anaerobic digester utilizes the waste to extract renewable biofuels and by-products. The anaerobic digestion process can be proved as a boon to the developing and underdeveloped countries. The widespread application of the process can help these countries to eradicate the major problem of waste management, sanitation, health, bio-fertilizer production, and renewable energy. One of the major products of anaerobic digester (AD) is methane (Li et al. 2015). As biomethane is the greenest of all the biofuels, it is going to be reclassified as "super low-carbon fuel" from "low-carbon fuels" for decentralized system (Fig. 1) (Nguyen et al. 2015).

In 1776, Volta identifies that the anaerobic digestion can potentially convert organic matter to methane (Sowers 2014). In 1981, *Cosmos* a French journal cited "Mouras Automatic Scavenger," the first anaerobic chamber to treat domestic

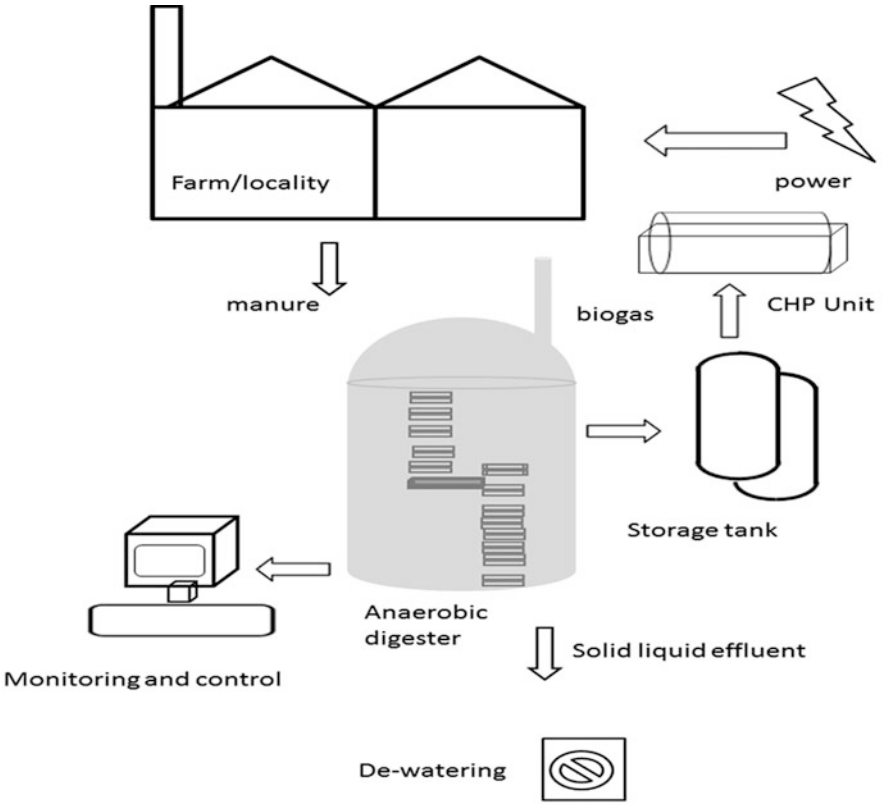

Fig. 1 Anaerobic digester-based biogas plant for decentralized power generation

wastewater. In 1895, Donald Cameron built a septic tank based on the model of Mouras Automatic Scavenger in Exter, England. The application of anaerobic process was limited till 1950, due to lack of the essential knowledge of the anaerobic process. In 1950, Stander identified the relevance of solid retention time (SRT) for anaerobic digestion of wastewater. This development has directed to the advancement of the high-rate anaerobic reactor. The progress of high-rate anaerobic reactor established the application of anaerobic process, particularly for biogas recovery and wastewater treatment (Li et al. 2015).

Methanogenesis is the process of formation of methane by some microorganisms commonly known as methanogens under anaerobic conditions (Venkata Mohan et al. 2011, 2013). Methanogens belong to the kingdom Archaea and are the most diverse group among the known members of the domain. These microorganisms drew the attention of the whole world due to their significant contribution to the global methane emissions and their wastewater treatment ability. Methanogens can produce methane using different organic and inorganic compounds such as carbon dioxide, acetate, etc. (Romero-Güiza et al. 2016). Methanogens can also utilize the waste products of different bacteria to produce methane. Methane production is a very versatile process which can utilize various organic and inorganic compounds to produce methane and carbon dioxide under anaerobic conditions (Kumar et al. 2014). The breakdown of the organic compounds is facilitated by a cascade of bacterial degradation enzymes by various groups of bacteria such as acidogens, acetogens, methanogens, etc. The methanogens are the most common microbes responsible for the reduction of the carbonaceous material to methane. They are the microbes which can thrive in anaerobic conditions such as bottom of the water bodies, paddy fields, dumping grounds, or in specialized environments—rumen bacteria to produce methane using organic matter (Kumar et al. 2016). The rumen bacteria form a symbiotic environment to a staggering amount of methane from cellulose. These bacteria produces 4×10^5 tonnes of methane daily (Ge et al. 2016).

2 Biochemistry of Methane Generation

The isolation and culturing of methanogens is very difficult as most of them are strict anaerobes. So, even a very small contamination of oxygen can ruin the culture. The anaerobic culture requires specific techniques such as pressurized culture vessels for liquid cultures and roll tubes for solid cultures (Ahring 2003). These techniques were compiled by Balch et al. (1979) in his review paper (Balch et al. 1979).

The primary fermentation, i.e., breakdown of macromolecules such as carbohydrates, proteins, lipids, etc. results in the formation of acetate, formate, carbon dioxide, and hydrogen (Chandrasekhar et al. 2015a, b). The methanogens produce methane by mainly two pathways: carbon dioxide reduction and acetate conversion. But methanogens can also convert formate, methanol, methylamines, and CO into methane (Chandrasekhar and Venkata Mohan 2014a, b; Stamatelatou et al. 2014).

2.1 Carbon Dioxide (CO_2) Reduction

The reduction of CO_2 is studied extensively by the researchers. The pathway uses many C1 coenzymes which are unique to methanogens. The conversion of carbon dioxide to methane occurs through many intermediate stages which begin with the conversion of carbon dioxide to formyl, methenyl, and methylene and ends with the formation of methane from methyl stage. The conversion of carbon dioxide to methane is facilitated by many C1 coenzymes which are mostly unique to methanogens. These coenzymes are tetrahydromethanopterin (THMP), methanofuran, and coenzyme M. THMP is similar to the eukaryotic pterins, while the latter two are unique to methanogens (Sebola et al. 2014).

The C1 group is passed in a bucket bridge fashion to a coenzyme as it is consecutively reduced. The first stable compound in the pathway is formyl-methanofuran. The formyl-methanofuran transfers the formyl group to TMHP in the presence of a formyltransferase. The formyl group is then converted to methenyl group by a cyclohydrolase named as 5,10-methenyltetrahydromethanopterin cyclohydrolase (Zinder 1990). The reduced coenzyme F420 donates two electrons to the methenyl group which results in formation of methylene group. The methylene group conversion is facilitated by an oxidoreductase enzyme named as methylene-tetrahydromethanopterin: coenzyme F420 oxidoreductase. The methylene is further converted to methyl group which forms a complex with coenzyme M. The conversion of methyl coenzyme to methane involves four enzyme complexes out of which only two are known till date. The known enzyme complexes involved are CoM-S-S-HTP and *N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine (H-S-HTP). The reactions intricate in the pathway are mentioned in Table 1. The conversion of carbon dioxide to methane accounts of a net Gibbs free energy of −130.4 kJ/mol (O'Flaherty et al. 2006).

Table 1 Reaction sequences for the anaerobic digestion of complex organic matter

Steps	Function	Microbiomes
Hydrolysis	Conversion of suspended organic matter, proteins, carbohydrates, and lipids to amino acids, sugars, and fatty acid	Fermentative bacteria (*Bacillaceae*, *Lactobacillaceae*, *Enterobacteriaceae*, etc.)
Acidogenesis	Conversion of amino acids, sugars, and fatty acid to intermediate products, C3, or higher organic acids like propionate and butyrate	*Clostridia*
Acetogenesis	Conversion of intermediate products, propionate, and butyrate to acetate, hydrogen, and CO_2	
Methanogenesis	Conversion of acetate and hydrogen to methane	Acetotrophic methanogen, hydrogenotrophic methanogen

2.2 Acetate Conversion

Early researcher proposed that the acetate is first completely oxidized to carbon dioxide. The carbon dioxide produced was then converted to CH_4. The carbon dioxide reduction theory was proved false by using ^{14}C-labeled acetate. The results showed that most of the methane was resulting from the methyl group ($–CH_3$) and little from the carboxyl (–COOH) carbon. Further, the hydrogen atom of $–CH_3$ was replaced by the deuterium atoms in other study to show that the intact $–CH_3$ is transferred to CH_4. The methanogens cleave acetate into $–CH_3$ which is reduced to CH_4 (Choong et al. 2016).The electron is derived from the oxidation of –COOH to CO_2. The pathway is mainly studied in *Methanosarcina* and can also be used to explain the acetate conversion in *Methanothrix* with some modifications. The first step involves the activation of acetate by converting it into acetyl-CoA with the expense of an ATP. The reaction is nonspontaneous with a Gibbs free energy value of +13 kJ/mol. The activation to acetyl-coA is followed by decarbonylation which is catalyzed by carbon monoxide dehydrogenase (CODH). CODH catalyzes the oxidation of carbonyl group to carbon dioxide and a molecule of ferredoxin is reduced in the process. The methyl group is transferred in the complex to corrinoid/iron-sulfur component and subsequently to coenzyme M (HS-CoM). The formed complex undergoes reduction to give methane (Gorris and van der Drift 1994). The last step involves the electrons from the reduction of ferredoxin but the electron transport chain is still unknown.

2.3 Other Pathways

The other pathway involves the conversion of CH_3OH and methylamines to CH_4 and CO_2. The CH_3OH is converted to CH_4 and CO_2. The $–CH_3$ of CH_3OH is transferred to coenzyme M (HS-CoM). The complex (methyl-coenzyme M) undergoes demethylation with 7-mercaptoheptanoyl-L-threonine phosphate (HS-HTP) which produces methane and the heterodisulfide (CoM-S-S-HTP). The electrons of the heterodisulfide are replenished from the oxidation of CH_3OH. The conversion of methylamines is not studied in details, but the researchers suggest that the conversion is analogous to CH_3OH (Gujer and Zehnder 1983).

3 Microbiology of Methane Generation in AD

The anaerobic digestion process is the sequential degradation of complex substrates to CH_4 (Table 1). It can be broadly divided into three steps on the basis of the different microbial consortium. The microbial consortiums involved in the process are acidogenic bacteria, obligate hydrogen-producing acetogens, and two groups of methanogenic Archaeas (Gerardi 2003).

3.1 Fermentative Bacteria

The first stage of anaerobic digestion process is responsible for degradation of complex substrates such as carbohydrates, lipids, and proteins into simpler substances by facultative and obligate fermentation. The first step is facilitated by the anaerobic species which belongs to the family of *Enterobacteriaceae* and *Streptococcaceae* and the genera of *Clostridium*, *Eubacterium*, *Lactobacillus*, *Bacteroides*, *Butyrivibrio*, and *Bifidobacterium* (Novaes 1986). *Bacillaceae* is the predominant in digestive sludge along with the others such as *Enterobacteriaceae* and *Lactobacillaceae* (Kumar et al. 2015). *Clostridia* ferments the hydrolyzed products of proteins such as amino acids and peptides into volatile fatty acids (VFAs), hydrogen, ammonia, and carbon dioxide (Narihiro and Sekiguchi 2007).

This first step of fermentation consists of hydrolysis and acidogenesis. This step converts the complex substrates into oligomers or monomers, for example, proteins are converted to smaller peptides or amino acids. The hydrolysis of complex substrate is followed by the further degradation of these simpler substances into VFAs and other products such as methanol, CO_2, formate, etc. The common volatile fatty acids generated are acetate and butyrate (Venkata Mohan and Chandrasekhar 2011a, b; Chandrasekhar and Mohan 2012; Kumar et al. 2012). This is generally the fastest step in the process. The cell count of acidogens in the digesters is between 106–108 per ml (Noike et al. 1985). A low partial pressure of hydrogen favors the production of desired precursors of methanogenesis such as acetates, carbon dioxide, and hydrogen. While high partial pressure of hydrogen shifts the process toward formation of other organic intermediates such as acetate, butyrate, formate, propionate, etc.

3.2 Hydrogen-Producing Acetogenic Bacteria (2) and Acidogenesis

This bacterial group is responsible for the degradation of higher organic acids such as propionic acid, butyric acid, etc. and aromatic compounds such as benzoate, ethanol, and other products to carbon dioxide, hydrogen, and acetate (Eqs. 1–3). These reactions are not thermodynamically favorable with pure culture. However, with coculture it is possible to convert C3 or higher organic acid to acetate at certain condition.

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2 \quad \Delta G^O = 76.1 \text{ kJ} \quad (1)$$

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \quad \Delta G^O = 48.1 \text{ kJ} \quad (2)$$

$$CH_3CH_2OH + 2H_2O \rightarrow CH_3COO^- + H^+ + 2H_2 \quad \Delta G^O = 9.6 \text{ kJ} \quad (3)$$

In 1967, Hungate studied the relevance of production and consumption of hydrogen in anaerobic processes. The process of hydrogen consumption by methanogens maintains a low partial pressure of hydrogen which favors the acetogenesis, i.e., breakdown of organic compounds into hydrogen, carbon dioxide, and acetate. Acetogenesis is the secondary fermentation process which converts the VFAs and other intermediates such as alcohols, hydrogen, and CO_2 into methanogenic precursors (hydrogen and acetate). So, this is a very critical step for the methanogenesis (Hungate 1967). The acetogens can be further classified into two types on the basis of their prevalence in the reactor: obligate hydrogen-producing acetogens (OHPAs) and homoacetogens. OHPAs are the microbes which convert intermediate compounds like fatty acids, alcohols into acetate, CO_2, and H_2. But the oxidation of OHPAs is inhibited by the presence of the hydrogen, a metabolic product. The Gibbs free energy of the reaction is highly positive in the presence of hydrogen (Kotelnikova and Pedersen 1997). So, the process efficiency can be increased by the microbial consortium which can utilize the produced hydrogen. The best available option for syntrophy is methanogens, which can consume the produced H_2. Hence most of the OHPAs grow in syntrophic environment with methanogens.

3.3 *Homoacetogens*

Homoacetogenesis is an interesting biochemical pathway in anaerobic digestion. The acetate is the major end product in homoacetogenesis, which is considered as an essential precursor for CH_4 production. The answerable microorganisms are either autotrophs or heterotrophs. The autotrophic homoacetogens, the second group of acetogens, convert CO_2 and H_2 produced into acetate (Eq. 4). This group of microbes also helps in maintenance of little partial pressure of H_2 in the reactor. Homoacetogens can also utilize other substrates as a carbon such as carbon monoxide (Eq. 5).

$$CO_2 + H_2 \rightarrow CH_3COOH + 2H_2O \quad (4)$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad (5)$$

$$4HCOOH \rightarrow CH_3COOH + 2CO_2 + 2H_2O \quad (6)$$

$$4CH_3OH + 2CO_2 \rightarrow 3CH_3COOH + 2CO_2 \quad (7)$$

The heterotrophic homoacetogens can utilize methanol and formate as a carbon source and produce acetate as an end product (Eqs. 6 and 7). In 1986, Novaes isolated two mesophilic homoacetogens: *Acetobacterium woodii* and *Clostridium aceticum*. In 1981, Zeikus observed that there is no accumulation of H_2 and CO_2 during the growth of homoacetogens. The studies have shown that the

homoacetogenesis is a rapid process (Diekert and Wohlfarth 1994). Even the Gibbs free energy that was found close to methanogenesis suggests competition for hydrogen and other electron donors (Eqs. 8 and 9).

$$H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O \ \text{(homoacetogenetion)} \ \Delta G^O = -104.6\,\text{kJ} \tag{8}$$

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \quad \text{(methanogenetion)} \quad \Delta G^O = -135.6\ \text{kJ} \tag{9}$$

3.4 Methanogen and Methanogenesis

Methanogenesis is considered as one of the major rate-limiting steps in the anaerobic digestion. The methanogens are found abundantly in anaerobic surroundings such as ponds, marine sediments, swamps, and lakes. The methanogenic precursors produced in the second step are utilized by the methanogens to produce methanogens (Archer and Harris 1986). The methanogens are obligate anaerobes which are further categorized into two types based on the substrate utilization: acetoclastic and hydrogen-utilizing methanogens. Acetoclastic methanogens are the major producers of methane which is close to 70% of the methane produced. They belong to mainly two genres: *Methanosaeta* and *Methanosarcina*. Generally, *Methanosarcina*, a spherical shaped methanogen, forms large packets which consist of coccoid (spherical) cell units. *Methanosarcina* can utilize various substrates such as H_2/CO_2 (eq), CH_3OH (eq), and methylamines (eq). The doubling time of *Methanosarcina* on acetate is 1–2 days (Eq. 11), while *Methanosaeta*, a rod-shaped methanogen, can only grow on acetate and their doubling time is 4–9 days (Conklin et al. 2006). So, CH_3COOH concentration greater than 1 mM favors the growth of *Methanosarcina*, and lower concentration favors *Methanosaeta's* growth. As compared to other bacteria, the growth rates of acetotrophic methanogens are slow which limits the anaerobic process and may result in the acetic acid accumulation.

$$4HCOO^- + 2H^+ \rightarrow CH_4 + CO_2 + 2HCO_3^- \quad \Delta G^O = -127\ \text{kJ} \tag{10}$$

$$CH_3COO^- + H_2O \rightarrow CH_4 + 2HCO_3^- \quad \Delta G^O = -28\ \text{kJ} \tag{11}$$

Hydrogen-utilizing methanogens consume hydrogens for the reduction of the process intermediates such as methanol, formate, carbon dioxide, and methyl amines to CH_4, the final product of anaerobic digestion. These microbes constitute the remaining 30% of the methane produced by the anaerobic process (Eq. 12). These are chemolithotrophic autotrophs, which derives both energy and carbon from inorganic chemicals (Ferry 2012).

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \quad \Delta G^O = -135.6 \text{ kJ} \tag{12}$$

Although biomethane production occurs via two major pathways—hydrogenotrophic methanogenesis via CO_2 reduction and acetotrophic/acetoclastic methanogenesis—there exists a third one, methylotrophic pathways. Methanol; mono-, di-, and trimethylamine; and dimethyl sulfide (reaction is a major substrate for methylotrophic methane generation where $-CH_3$ is transferred to a methyl carrier and reduced to CH_4 (Eqs. 13–15)).

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad \Delta G^O = -103 \text{ kJ} \tag{13}$$

$$4CH_3NH_2 + 2H_2O + 4H^+ \rightarrow 3CH_4 + CO_2 + NH_4^+ \quad \Delta G^O = -102 \text{ kJ} \tag{14}$$

$$(CH_3)_2S + H_2O \rightarrow 1.5CH_4 + 0.5CO_2 + H_2S \quad \Delta G^O = -74 \text{ kJ} \tag{15}$$

4 Environmental Factors

As mentioned in the previous sections, anaerobic process is strongly impacted by the environmental factors. The anaerobic process requires a stricter control over the environmental parameters than the aerobic process. The impact of environmental parameters on the anaerobic process is measured by methane yield as methanogenesis is the rate-determining step of the anaerobic process. Methanogenesis, a biological process, is strongly impacted by environmental factors such as toxicity, temperature, pH, type and concentration of feed, nutrients, metal toxicity, and others. A brief outline of the impact of the abovementioned factors is described here.

4.1 Temperature

Methanogens can produce methane in a temperature range of 10–60 °C. The rate of methane production is very slow at 10 °C which attains saturation at a temperature of 37 °C. The anaerobic digestion can be segregated in three groups: psychrophilic (10–20 °C), mesophilic (20–40 °C), and thermophilic (40–60 °C) digestion. The microbial growth and the conversion process are strongly influenced by temperature. So, psychrophilic digestion requires a long retention which results in large reactor volume, and mesophilic digestion requires a small reactor as compared to the psychrophilic. Thermophilic, being a special case, can be utilized for wastewater treatment which is discharged at high temperature. The only methanogen which shows higher specific methanogenic activity is hydrogenotrophic methanogens with no change in the most probable number (MPN) at 65 °C, compared to

55 °C. While the number and activities of other microorganisms were considerably reduced (Sowers 2014).

The temperature requirement can be expressed mathematically using Arrhenius expression:

$$r_t = r_{30}(1.11)^{(t-30)} \tag{16}$$

where "t" is the temperature in °C and r_t, r_{30} are rates of digestion at temperature t and 30°C, respectively. On the basis of the above equation, the optimum digestion rate decreases by 11% for every 1 °C decrease in optimum temperature. Temperature severely impacts the decay rate, maximum specific growth rate, growth yield, and half-velocity rate constant. So, the temperature effect on methane yield is the cumulative impact on different growth kinetics parameters. The rate of methane generation in thermophiles is independent of temperature between 50–70 °C. Hence, Water Pollution Control Federation (WPCF) recommended that the anaerobic process should be designed in such a way that the variation in process temperature should not exceed 0.6–1.2 °C/day (Griffin et al. 1998).

4.2 Operating pH

The pH in an anaerobic digester changes with the onset of the production of VFAs by acidogenic bacteria in the early stage of the process. But a further decrease in pH is observed in the latter stage due to the consumption of VFAs by the methanogens. Most of the anaerobic methanogens grows in neutral pH range (6.5–7.5). The methane production is inhibited at low pH which develops in anaerobic reactors due to the generation of volatile fatty acid intermediates (Venkata Mohan and Chandrasekhar 2011a). So, it is essential to maintain the pH for optimal productivity. Hydrogen bicarbonate is used to keep the pH in the neutral pH range. The optimal pH for the growth of acidogens lies in the range of 5.5–6.5, while that of methanogens ranges in 7.8–8.2. So, the challenge is to select a pH range at which both the microbial groups can coexist. The optimal pH for the coexistence of both the microbial groups is 6.8–7.4. Hence, it is advisable to uphold the pH close to the neutral (Griffin et al. 1998).

4.3 Alkalinity

The organic waste which contains high nitrogen can significantly contribute to the alkalinity. The primary constituent of this wastewater is proteins which on degradation generates ammonia. The ammonia reacts with the carbon dioxide present in

the digester to generate bicarbonate which is mainly responsible for alkalinity in the digester. The biochemical reaction is as mentioned below (Eqs. 17 and 18):

$$\mathrm{RCHNH_2COOH + 2H_2O \rightarrow RCOOH + NH_3 + CO_2 + 2H_2} \tag{17}$$

$$\mathrm{NH_3 + H_2O + CO_2 \rightarrow NH_4^+ + \underset{\text{(alkalinity)}}{HCO_3^-}} \tag{18}$$

However, treatment of sulfate-/sulfite-rich wastewater under anaerobic conditions also generates alkalinity due to sulfate/sulfite reduction (Eqs. 19 and 20). Theoretically, 1000 mg of SO_4 reduction generates 1040 mg of alkalinity as calcium carbonate ($CaCO_3$) (Sambo et al. 1995).

$$\mathrm{H_2 + SO_4^{2-} + CO_2 \rightarrow HS^- + HCO_3^- + 3H_2O} \tag{19}$$

$$\mathrm{CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^-} \tag{20}$$

The biogas produced from sulfate-/sulfite-rich wastewater has a significant amount of H_2S in it. H_2S or hydrogen sulfide is hazardous and corrosive by nature due to its highly acidic property, and therefore it is imperative to quench this "sour gas." This process is known as "gas sweetening." The gas sweeting is done using an iron sponge which has a bed of wood chips and hydrated oxide. This bed removes the H_2S present in the biogas streams. Amine plants can remove both H_2S and CO_2 from liquid hydrocarbons and natural gas. The process involves both chemical reactions and adsorption. The H_2S removal process should meet the "pipeline quality gas" standards which is less than 0.25 grains of H_2S (Díaz and Fdz-Polanco 2012).

4.4 Oxidation-Reduction Potentials

Many researchers have reported that the growth of obligate anaerobes requires a culture with an ORP value in the range −200 to −350 mV at pH 7. The methanogens can grow well in an extremely reducing environment, which has an ORP of −400 mV. Hence, the culture media for methanogens has a good amount of reducing agents such as cysteine, sulfide, or titanium III to maintain the ORP at a suitable value (Prasad and Prasad 2012).

4.5 Type and Concentration of Feed

The anaerobic digesters have evolved a lot with the research in the last decades. The present digesters are compatible to various types of feed such as agricultural wastes, industrial wastes, algal biomass, etc. as compared to the trivial digesters which can

use only sewage sludge and animal wastes (Tijani et al. 2015). The constituents of feed vary with the type of waste used, for example, paper industry waste is quite rich in organic matter, food waste is rich in soluble organics, etc. The anaerobic digestion is seriously affected by the presence of many materials such as sand, plastics, and glass which results in the process failure if present in high concentration (Kimari et al. 2015).

The concentration of feed also plays an imperative role to decide the productivity of the reactor. The increase in solid content of the slurry decreases the rate of CH_4 production. The study done by Fernandez et al. shows that the CH_4 production was reduced by 17% with the increase of solid content from 20% to 30% (Ge et al. 2016).

4.6 Nutrients

The major nutrients required by the microbes are carbon, hydrogen, nitrogen, phosphorus, and sulfur. Most of the waste materials lack nitrogen and phosphorus. The waste is characterized mainly by C/N ratio which is the ratio of carbon and nitrogen present in the waste. The productivity of the digester can be enhanced by adding appropriate nutrients in optimum amount. We can also mix different waste to optimize the nutrient constituents of the digester (Jain et al. 2015).

4.7 Metal Toxicity

All microbes need trace elements and nutrients for waste stabilization, but these elements are not directly responsible for waste stabilization. These elements are the essential components for growth and synthesis of cells. These elements also provide optimal physicochemical conditions for microbial growth. Some metals such as Ni, Fe, Mo, and Co are also used by methanogens for growth on H_2 and CO_2 (Choong et al. 2016). The metals are required by the microbes for growth in an optimum amount. But these metals can also be toxic to microbes if present in high amounts. The presence of metals such as Na^+, K^+, Ca^{2+}, etc. in high concentrations is toxic to methanogens. These metal salts can disturb the tonicity of the bacterial membrane which results in very deleterious effects (Schmidt et al. 2014).

5 Reactor Design

The selection of optimum reactor configuration is the most important part of any process. The slow reaction rate of biomethanation demands strict control parameters for reactor design. The rate-limiting step of any process determines the

optimum reactor design which is methanogenesis in anaerobic digestion. The selection of reactor configuration is by high HRT/SRT ratio, which prevents the washout of methanogens. The most crucial parameter is sludge retention time (SRT) which directly impacts growth rate of the methanogens and hence productivity of the process. There are many other parameters which need attention for stable methanogenesis. The other process parameters of an anaerobic process are hydraulic retention time (HRT), process temperature, C/N ratio, organic loading, pH, mixing, etc.

5.1 Sludge Retention Time (SRT)

The SRT is defined as the ratio active biomass in the system to the production of active biomass in the system.

$$\text{SRT} = \frac{V \times X}{Q_{\text{out}} \times X_{\text{out}}}$$

where V is the volume of reactor, X is the reactor cell mass, and X_{out} is the cell mass flowing out of the reactor with a flow rate Q_{out}. If SRT is less, the methanogenesis will not take place which results in pH decrease due to accumulation of VFAs. While very high SRT can result in a nutrient deficiency in the reactor. So, an optimum SRT of 15 days is required for the production of methane in an anaerobic digester.

5.2 Hydraulic Retention Time (HRT)

HRT is defined as the ratio of reactor volume (V) and inflow rate in the reactor (Q).

$$\text{HRT} = \frac{V}{Q}$$

HRT of the process depends on the feed type and concentration and temperature. High HRT can be used for low feed concentration, while a high HRT is required for high feed concentration. If process temperature is high, then the rate of reaction increases and HRT decreases for the same yield and vice versa.

5.3 Temperature

As described in Sect. 4.1, methanogens can grow in a range of temperature 10–60 °C. The optimal temperature for methane production is 37 °C. The conventional reactors are designed to function in mesophilic conditions. The temperature in such reactors is maintained in a range between 37 °C ± 2 °C. The reactors operating under mesophilic conditions require less energy input and are more stable than thermophilic reactors (Wang et al. 2014). While the CH_4 production rate of mesophilic is more than the psychrophilic reactors. So, the process temperature in an AD has to be strictly maintained throughout the operation time (Abdelgadir et al. 2014).

5.4 Carbon/Nitrogen Ratio

The C/N ratio gives the relative amount of nitrogen and carbon present in the reactor. The carbon is required by all the cells, but nitrogen is also a crucial factor for synthesis of proteins in the cell. The optimal C/N ratio is 20–30. The substrates with a very high C/N ratio are deficient in nitrogen and vice versa. The imbalance in C/N ratio can result in high VFA and total ammonia formation. Both the intermediates are the potential inhibitors of the methanogens. So, it is very essential to maintain the C/N ratio in the anaerobic digester (Wang et al. 2014).

5.5 Organic Loading Rate

Organic loading rate (OLR) defines the amount of organic substrate loaded per unit volume of the reactor.

$$\mathrm{QLR} = \frac{Q \times C_{\mathrm{VS}}}{V}$$

where C_{VS} is the concentration of volatile solids. Lower OLR results in a large reactor volume, while a higher OLR requires a small reactor. But higher OLR requires higher HRT and it can also lead to the overloading of the reactor. So, the reactor design should balance the HRT and OLR (Chandrasekhar and Venkata Mohan 2012).

5.6 pH

The pH of the digester lowers due to generation of many intermediates such as VFAs during digestion. The AD also involves a consortium of microbes which grows at different temperatures, for example, acidogens grow optimally in the pH range of 5.5–6.5 while acidic pH is toxic to methanogens. The biggest reason of the anaerobic digester failure is acid accumulation. So, the reactor has to be well equipped to maintain the pH in the desired range (Abdelgadir et al. 2014).

5.7 Mixing

The fermentation media should be mixed properly to maximize the degradation of the substrate by enzymes produced by the microbes. A proper mixing regulates a better temperature and pH control. It also maintains a uniform heat and mass transfer throughout the reactor. Mixing can be done in various ways such as recirculation of different reactor's contents, mechanical mixing, etc. The mixing time and speed have to be optimized because overmixing can led to the degradation of microbes present in the anaerobic digester (Jain et al. 2015).

5.8 Ideal Anaerobic Reactor

Hence the ideal anaerobic digester should have following characteristics: optimal temperature and pH control, optimum balance between HRT and OLR, and a proper mixing time and speed. The disruption in the anaerobic digestion process due to acid accumulation can be solved using two stage anaerobic digestions. The digestion process is divided into two processes in two separate digesters. The first process involves hydrolysis and acidogenesis, while the second process involves acetogenesis and methanogenesis. The anaerobic digesters can be classified in three categories on the basis of the reactor design: low-rate (long hydraulic retention time), high-rate (short hydraulic retention time), and tubular reactor (Skiadas et al. 2003).

5.8.1 Low-Rate Reactors

The long hydraulic time is required for the waste treatment from streams such as solid wastes and slurry, while wastewater treatment requires short hydraulic retention time. The low-rate systems are batch, plug flow, and continuous reactors. The low-rate digestion process can be further classified into two types on the basis of number of steps in the process: one step and multiple steps mainly two steps. The

digesters can be categorized in two categories on the basis of the dry matter present in the slurry: wet (dry matter less than 20%) and dry digester (dry matter between 20% and 40%).

5.8.2 High-Rate Reactors

The high-rate systems are further categorized into systems with fixed beds and systems with suspension culture. The fixed-bed system consists of fixed bacterial biofilms on a solid support, while in the latter the microbial mass is retained by settling (internal or external). The internal settling can be done by simply with the help of gravity, while external settling can be done by using external means such as a filter at the outlet. The low-rate systems are plug flow, batch, and continuous stirred tank reactors, while the high-rate systems are fluidized bed and upflow anaerobic sludge bed (UASB) reactors.

The UASB reactors are the most common reactor used by both industries and research. The UASB reactors function at solid content of 4–15% and a HRT of 0.5–12 days. The UASB reactors can be used at higher organic loading rate as compared to other high-rate reactors (Bal and Dhagat 2001).

5.8.3 Tubular Reactors

These reactors are used to produce methane at high altitude at process temperature between 10 and −20 °C (psychrophilic conditions). These are mainly household digester of volume ranges between 2.4 and −7.5 m^3. These reactors are used to treat animal wastes with a HRT of 60–90 days. The low-cost tubular reactors have following problems: HRT and biogas pressure. The HRT is calculated by the reactor size not by the pit dimensions. So, the HRT changes during the process depending on the change of volume of the cylindrical bag used as a reactor. Similar is the case with the biogas pressure which also decreases due to reduction in HRT (Lettinga 1995).

The anaerobic digester has following basic components: digester vessel, biogas utilization system, premixing tank, effluent spreading, or distributing system.

The digesters can be classified in two categories: batch and continuous. The batch digesters are very simple to build and operate. The operation of batch digester is having the following steps: (1) loading the digester with the waste material to be digested, (2) the digestion process, (3) and effluent removal. The process is repeated after the completion of these steps. On the other side, the continuous digesters are fed regularly with the waste material. The material is infused into the digester either by flow of the feed or by mechanical methods such as pumps. These digesters can provide a continuous supply of biogas without any lag time due to loading and unloading of the effluent. The continuous digester can be further classified into: horizontal tanks, vertical tanks, and plug flow systems (plug flow digesters and multiple tank systems) (Abdelgadir et al. 2014). Continuous digesters can be used

Table 2 Basic types of reactors used in anaerobic biogas generation

Reactor type	Synonyms
Attached-based growth	
Fixed bed	Fixed film, filter, submerged filter, stationary fixed bed
Moving bed	Rotating discs, rotating biological contactor (RBC)
Expanded bed	Anaerobic-attached film expanded bed
Fluidized bed	Carrier-assisted contact process
Nonattached biomass	
Recycled flocks, sludge blanket, digester	Contact process, upflow anaerobic sludge blanket (UASB), clarigester type

for large-scale production of biogas, but they require high maintenance and operating cost and a more sophisticated design as compared to the batch digesters. Even though anaerobic digesters have been used for sewage stabilization for many years, the use of these digesters is a recent phenomenon. The increasing use of the anaerobic digesters is due to the new reactor designs and better fundamental understanding of the anaerobic process. These new digesters can retain a much higher biomass which has substantially decreased the lag time of the process. The biomass retention technology can be further categorized into two groups: suspended growth (sludge blanket reactors) and attached-based growth (fluidized digesters) (Bal and Dhagat 2001). Different types of anaerobic reactor types used in industries are tabulated elsewhere (Table 2). Schematic representation of these reactors is illustrated in Fig. 2 (Skiadas et al. 2003).

6 Feedstock Used in AD

With the advancement of new anaerobic technology, the biogas can be produced using various feedstock such as plant extract like jatropa, poplar, miscanthus, seashore, sorghum, spartina, switch grass, camelina, etc. The feedstock mentioned above can be grown on marginal lands with very little fertilizer and water (Kimari et al. 2015). Apart from the agricultural feedstock, biogas can be produced from food processing wastes such as corn cobs, fruit waste, cheese whey, nut shells, rice hulls, restaurants waste, sugar waste, etc. Biogas can also be produced from the agricultural wastes (Ge et al. 2016).

6.1 Environmental Advantages of AD

Anaerobic digestion is a sustainable process to get rid of different waste generated and energy recovery from the waste. The bio-CH_4 can be utilized as a potential fuel source for an onsite power plant (Fig. 1). This can also benefit the facility for

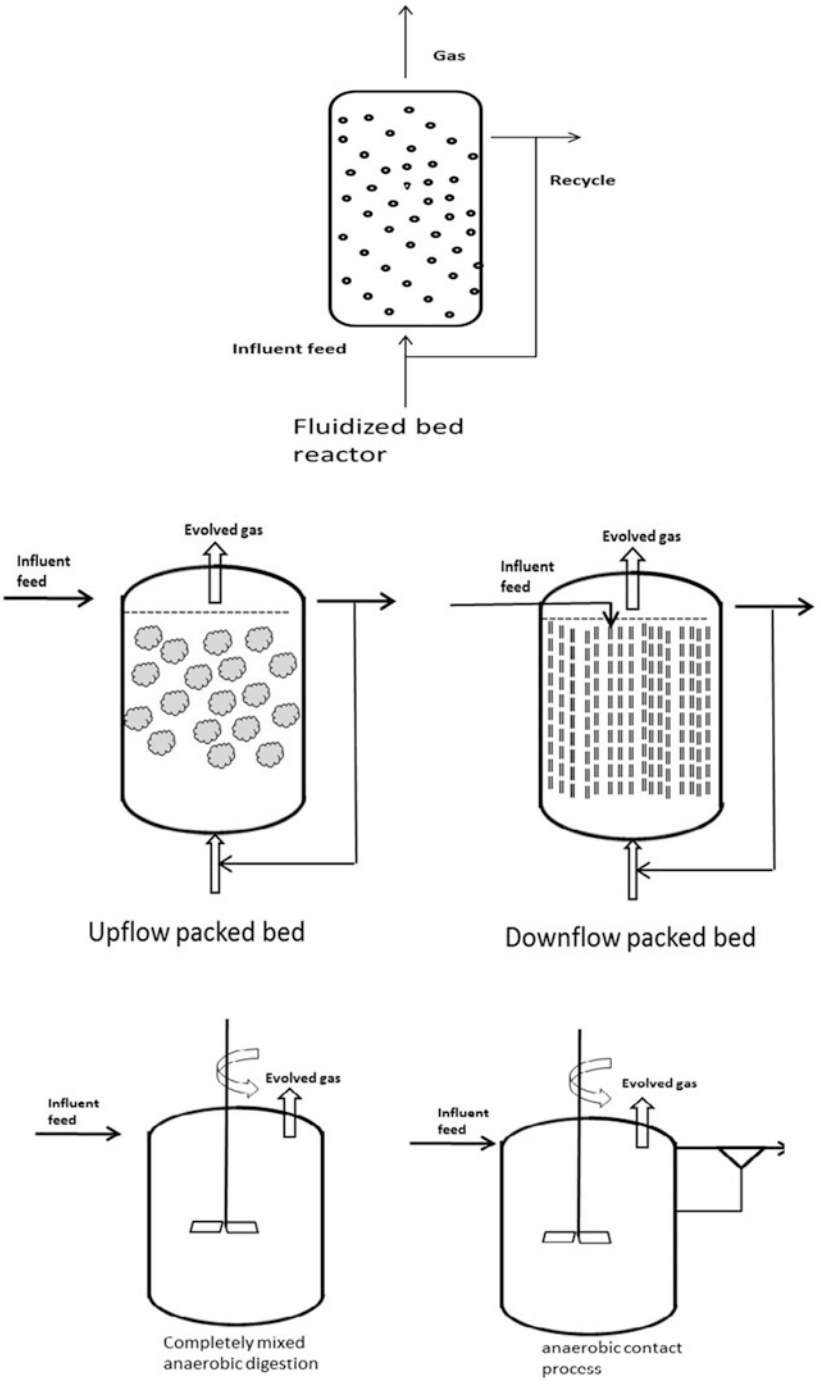

Fig. 2 Typical reactor configurations used in anaerobic wastewater treatment

additional revenue streams in various forms such as carbon dioxide credits, renewable energy credits, and/or greenhouse gas emission credits. The anaerobic process can reduce the BOD and COD by 50% approximately. Anaerobic digestion also reduces the phosphorous and nitrogen content of the wastewater which are mainly responsible for eutrophication in lakes or ponds. The process also reduces odor, surface water pollution, and groundwater pollution (Li et al. 2015).

6.2 Bottleneck of AD

Even though the anaerobic digestion has many benefits, it cannot treat all types of wastewater. The anaerobic digester is a good option for biomethane production, but they also have few bottlenecks such as the slow start-up time, large reactor volume, hydrogen sulfide generation, and ammonia inhibition (Ferry 2012).

6.2.1 Ammonia Inhibition

Ammonia is formed in anaerobic digestion due to degradation of nucleic acid, proteins, and urea. The ammonia is present as two forms in aqueous solutions: ammonium ions (NH_4^+) and free ammonia (NH_3). The optimum amount of ammonia (concentrations less than 200 mg/L) is helpful to anaerobic digestion process that helps to maintain the pH of the digester. But in most of the digesters the excess ammonia is the main inhibitor of the methanogenesis. The indicators of excess ammonia are increased production of intermediates such as VFAs and decrease in production rate of methane. There are many mechanisms proposed by researchers to explain ammonia inhibition: enzymatic reaction inhibition, change in pH, and adding further burden to maintenance energy. The methanogens are highly sensitive to increase in ammonia concentration among all the microbes present in anaerobic digesters. But the methanogens can be adapted to the change in ammonium concentration. This tolerance can be enhanced by change in metabolic pathways or shift in the methanogenic population (Abouelenien et al. 2010).

Free ammonia is highly permeable to the bacterial cell and hence the main cause of ammonia inhibition. Free ammonia diffuses into the microbial cells resulting into potassium deficiency or proton imbalance. The free ammonia changes to ammonium ion inside the cell and absorbs hydrogen ions present in the cell. Hence, free ammonia compels cell to expend more energy to maintain the ionic balance inside the cell. This can cause the inhibition of enzymatic reaction and increases the energy expenditure (Strik et al. 2006). Ammonia can inhibit the anaerobic digestion process at different levels: steady-state inhibition and accumulation of intermediates such as VFAs which can decrease the pH to very low value. The decrease in pH can further aggravate the problem (Wang et al. 2014).

6.2.2 H_2S Generation

Sulfate is a very commonly found in industrial and other wastewater. The sulfate present can be reduced by the sulfate-reducing bacteria (SRB) present in the anaerobic digester. There are the two types of SRB: complete oxidizers and partial oxidizers. The complete oxidizers reduce CH_3COOH to CO_2 and bicarbonates, while the partial oxidizers convert other compounds such as lactate to CO_2 and acetate. SRB inhibition can be divided into two stages: primary and secondary inhibition. Primary is a type of competitive inhibition in which SRB competes with other microbes for substrate. This competitive inhibition results in the suppression of methane production (Peu et al. 2012). Secondary inhibition is due to the toxic effect of sulfide on SRB and other microbial groups including methanogens (Chattanathan et al. 2014).

SRB can compete for substrate with almost all kind of microbes present in the anaerobic digester except microbes present in hydrolytic stage. As SRB are not equipped to degrade the complex substrates such as glycogen, lipids, starch, protein etc., SRB have a very high affinity toward propionate which is a very crucial intermediate formed by the acetogens. SRB can also compete for other intermediates such as butyrate and ethanol.

The studies done to establish the toxic effect of sulfides on microbes and its nature are not very conclusive. Some studies supports that H_2S is a highly toxic form of sulfides, which can diffuse into the cell and forms disulfide and sulfide cross-links between the polypeptide chains which result in denaturation of the proteins. H_2S can also affect the sulfur metabolism and sulfur linkages in various coenzymes. Despite of so many toxic effects, sulfur is a required nutrient by the microbes. The optimal concentration of sulfur in anaerobic digestion differs from 0.001 to 0.025 g/L. However, an inhibitory concentration also varies in a very broad range from 0.1 to −0.8 g/L dissolved sulfur and 0.05 and −0.4 g/L of undissociated hydrogen sulfide approximately (Kang et al. 2010).

7 Conclusions

The technology of anaerobic digestion has highly matured in the past decades. Now, production of methane by anaerobic digestion is used everywhere. The anaerobic digesters have also evolved to use various substrates such as industrial and agricultural wastewater, sewage sludge, household waste, kitchen waste, etc. The energy extraction from the waste can be further maximized by using efficient ways of conversion of waste to biomethane, and it will also depend on the efficient ways of energy utilization. The anaerobic digestion process requires less space and energy. The methane production by anaerobic digestion can be done using simple technology. The process also leaves less amount of stable sludge which can be further used as manure or can be dewatered to form compact leftovers. The fuel

gas produced as the final product is a mixture of CH_4 (55–75% v/v) and CO_2 (25–45% v/v). The calorific value of the mixture produced ranges between 22 and −30 MJ/Kg. The amount of gas produced by the process depends on the concentration of the digestible organics present in the waste and the process parameters such as pH, temperature, etc. The anaerobic digestion process has many applications such as disposal of different industrial and agricultural wastes, sewage purification, production of organic manure, conversion of biomass to energy, etc. The biogas produced by the process also has many applications such as electricity production using fuel cell, cooking fuel, fuel in many industries, etc. Despite much advancement, the biomethanation still have many areas to improve such as slow rate. The main bottleneck which requires improvements are biogas productivity, high capital investment, modular design, and operating cost.

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Laccases: Blue Copper Oxidase in Lignocellulose Processing

Dayanand C. Kalyani, Jogi Madhuprakash, and Svein Jarle Horn

Abstract The depletion of fossil fuel reserves, environmental pollution, and climate change are driving the search for clean carbon-neutral fuels. Lignocellulosic biomass is considered as a promising feedstock for production of bioethanol and biochemicals. The overall potency or utilization depends on the effectual hydrolysis of lignocellulose; however, removal or deconstruction of the lignin polymer could be a key step in the process of biomass to monomeric fermentation sugars but remains challenging. Laccases (EC 1.10.3.2) are copper-containing oxidoreductases that have been investigated for use as a pretreatment of lignocellulose and might have a potential to remove phenolic compounds derived from lignin. This chapter focuse on recent trends in ligninolytic green biotechnology and major advances within the application of laccases as a possible pretreatment strategy. Also, it discuss the negative roles of lignin within the processes of converting biomass to biofuels. Views and future directions to boost the biomass conversion process are also mentioned.

Keywords Laccase • Lignin • Laccase • mediators system • Catalysis

1 Introduction

In the twenty-first century, the demand for fossil fuels is increasing progressively beside improvements within the quality of life, inauguration of the economic revolution, and increase of the global population. It's long been recognized that the rise in the rate of fossil fuel consumption not solely ends up in decreasing fuel reserves; however, it conjointly includes an important adverse impact on the surroundings, leading to raised health risks and the threat of worldwide climate change (Panwar et al. 2011). Hence, there is a need to develop a sustainable alternative to fossil fuels. The usage of potential food resources in first-generation biorefineries has sparked intensive dialogue, due to the lack of land for food

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cultivation and large commitment of land required for biofuel production (Bugg et al. 2011). The food vs fuel dialogue encouraged the event of second-generation biofuel industry that uses lignocellulose biomass as feedstock. Lignocellulose biomass is one among the foremost rich sources of renewable energy and is principally composed of polysaccharide (40–50%), hemicellulose (20–40%), and polymer (20–30%).

Enzymatic hydrolysis reaction of lignocellulosic biomass is one of the key steps for sugar production and a requirement for succeeding conversion of sugars to biofuels and chemicals.

However, the presence of lignin in biomass forms a major obstacle for efficient utilization of cellulose and hemicellulose in biofuel production. The inhibition impact of lignin on enzymatic hydrolysis is classified into three classes: (1) Enzymes are adsorbed on lignin polymer through hydrophobic, static, and hydrogen bonding interactions. (2) Lignin blocks the accessible surface of carbohydrate polysaccharides through the physical and chemical blockage. (3) Soluble lignin derivatives such as phenolic compound can deactivate enzymes.

An appropriate pretreatment method is required to overcome this obstacle and makes polysaccharides easily available for enzymatic hydrolysis. To date, various pretreatment methods such as physical, chemical, and physicochemical are used to remove this obstacle (Sun et al. 2016). However, these pretreatment methods may generate inhibitory compounds that interfere with the enzymatic hydrolysis and succeeding fermentation processes.

There is an increasing interest to use biological pretreatments as an alternative physicochemical treatment (Kuijk et al. 2015; Lee et al. 2012). For the last decades, white-rot fungi have received a lot of interest because of their potential to reduce lignin content in wood and other lignocellulosics (Dashtban et al. 2010). Through an extracellular ligninolytic system, comprising a complex cocktail of oxidative enzymes, the substrate is oxidized by single electron withdrawals to create reactive radical intermediates. The substrate is then modified through continuous nonspecific and enzyme-independent chain reactions to produce various free radicals (Zhou et al. 2013). The enzymes responsible for degradation of lignin in white-rot fungi belong to four major groups: lignin peroxidases (LiP) (EC 1.11.1.14), manganese-dependent peroxidases (MnP) (EC 1.11.1.13), versatile peroxidases (VP) (EC 1.11.1.16), and laccases (Lac) (EC 1.10.3.2). In addition to LiP, MnP, VP, and Lac, other types of oxidative enzymes, together denominated auxiliary enzymes, have been reported to assist the lignin oxidation. These include aryl alcohol dehydrogenase, glyoxal oxidase, and pyranose oxidase, which have been reported to enhance the process through their peroxidase generating activity (Martinez et al. 2005; Ruiz-Duenas and Martinez 2009). The impact of the individual oxidases, as well as their possible interactions in the white-rot fungi degrading system, has not been fully elucidated. Published literature tend to deal with either single enzymes or a broth produced by specific fungal strains. The peroxidases (LiP, MnP, VP) are all dependent on hydrogen peroxide in order to be efficient enzyme catalysts in lignin oxidation. The hazardous effect of hydrogen peroxide prohibits the use of peroxidases in the food industry and severely complicates the utilization in

Table 1 Recent update of laccase application in biomass detoxification/delignification

Microbial laccase	Raw material	Pretreatment method/mediator	Role in processing	Remark	References
Trametes versicolor	Poplar and rice straw	Hot water treatment of rice straw at 220 °C, 54 min and dilute acid treatment of poplar at 160 °C, 30 min, using 1.0% (v/v) sulfuric acid; 1-HBT	Removal of phenolic and xylo-oligosaccharide inhibitors	Removal of 83% and 95% of the phenolic compounds in rice straw and poplar hydrolyzates, respectively; reduce HMF, furfural concentration in poplar hydrolysate by 25% and 27% without and with mediator, respectively	Kapoor et al. (2015)
Ganoderma lucidum 77002	Corn stover	Steam explosion using 0.8% H_2SO_4 (v/v) at 60 °C for 30 min	Removal of phenolic compounds	Upon laccase addition no significant effect on cellulase hydrolysis; nevertheless improve 10% ethanol yield	Fang et al. (2015)
Pycnoporus cinnabarinus	Pine, birch, and spruce	Steam explosion using SO_2 at 203 °C for 5 min	Detoxification	Laccase effective under more severe conditions and improve the CH_4 yields by 14.3 and 9.8%, in spruce and pine, respectively	Matsakas et al. (2015)
Yarrowia lipolytica SKU507	Populus balsamifera	Acid pretreatment (0.5% H_2SO_4 (w/w)) at 121 °C for 30 min	Removal of phenolic compounds	62% removal of phenolic compound at pH 5 helps to improve the saccharification yield by 43%	Kalyani et al. (2015)
Pycnoporus cinnabarinus	Wheat straw	Steam explosion at 210 °C, 2.5 min for delignification experiments and 220 °C, 2.5 min for detoxification assays; HBT, VIO, and ABTS	Leads to lignin polymerization; reduce the soluble phenols content	Lower glucose recoveries during saccharification, in contrast polymerization reduce the soluble phenols content and enhance the ethanol yield by 20%	Moreno et al. (2015)

(continued)

Table 1 (continued)

Microbial laccase	Raw material	Pretreatment method/mediator	Role in processing	Remark	References
Pycnoporus sanguineus UPM4	Oil palm empty fruit bunch	Dried at 60 °C and ground to 1 mm size followed by removal of extractive by ethyl alcohol and benzene (1:1 v/v ratio); ABTS, HBT	Delignification and detoxification	Pretreatment using ligninolytic enzyme-mediator systems (laccase-MnP-mediator) leads in increase of 16–17% of hydrolysis yield with approximately 30 g/L of sugar	Zanirun et al. (2015)
Pleurotus sp.	*Saccharum spontaneum* (wasteland weed)	Sun dry and chopping (0.2 mm size); Vanillic acid, ABTS, and methyl syringate	Delignification	Optimization led to 84.67% and 462.18 mg/g delignification and reducing sugar, respectively, with an increased crystallinity of 10.14%; delignification condition does not lead to changes in the structures of cellulose and hemicellulose	Rajak and Banerjee (2015)
Myceliophthora thermophila/ *Pycnoporus cinnabarinus*	Eucalypt (*Eucalyptus globulus*)	Ball milling, methyl syringate, HBT	Delignification	Decrease of guaiacyl and syringyl lignin units and aliphatic (mainly β-O-4′-linked) side chains, delignification (~50%) of eucalypt feedstock and increases (~30%) in glucose yields	Rico et al. (2015)
Escherichia coli (CueO)	Barley straw	Pretreatment by laccase alone	Delignification	Laccase fused with dockerin domain of a cellulosome system improved laccase activities and lead to 2.6 and 2.1-fold fold increase in the amount of reduced sugar and ethanol, respectively	Hyeon et al. (2014)

Trametes versicolor	Wheat straw	Acid pretreatment (2% H_2SO_4 at 126 °C for 1 h in a priorclave) followed by alkaline-peroxide extraction and laccase mediator system	Delignification	Combination of LMS treatments with an alkaline-peroxide extraction step increased the released glucose concentration following hydrolysis by up to 2.3 g L^{-1}	Heap et al. (2014)
Myceliophthora thermophila	Eucalypt (*Eucalyptus globulus*)	Ball milling, methyl syringate	Lignin is depolymerize and release from the sample causes the 50% reduction Kalson lignin	High release yield of glucose and xylose using relatively low doses of cellulases; 2D NMR and Py-GC/MS analysis shows the relative reduction in G lignin	Rico et al. (2014)
Trametes hirsuta	Spruce	Impregnation with SO_2 gas and steam explosion at 212 °C, 5 min; ABTS, HBT, TEMPO, and acetosyringone	Lignin modification	Decreased adsorption of cellulases on lignin and increasing hydrolysis yields, cellulose oxidation (at the C6 position) by laccase-TEMPO	Moilanen et al. (2014)
Commercial laccase	Corn stover	1 HBT	Delignification	Increased methane yield by 25% (352 CH_4/Vs)	Schroyen et al. (2014)
Pycnoporus cinnabarinus	Wheat straw	Steam explosion at 220 °C, 2.5 min	Detoxification	Shortened the yeast lag phase from 12 to 6 h and improved cell viability in SSF leads faster glucose consumption and ethanol production rates	Moreno et al. (2013a)
Pycnoporus cinnabarinus	Wheat straw	Steam explosion at 210 °C, 2.5 min	Detoxification	Phenols affected negatively the performance of *S. cerevisiae*; laccase pretreatment was improved hydrolysis and fermentation yield	Alvira et al. (2013)

(continued)

Table 1 (continued)

Microbial laccase	Raw material	Pretreatment method/mediator	Role in processing	Remark	References
Pycnoporus cinnabarinus	Wheat straw	Steam explosion at 210 °C, 2.5 min	Reduction of phenolic compound	Laccase supplement during fed-batch operational mode allows the fermentation at high dry matter (16%) with improving final ethanol concentrations and yields	Moreno et al. (2013b)
Ganoderma lucidum	Sugarcane baggase, barley straw and wheat straw	Steam explosion at 210 °C, 5 min	Detoxification	~30–46% in reducing sugar improvement	Sitarz et al. (2013)

other industrial applications. Besides being an additional financial expense, the corrosive action of hydrogen peroxidase is problematic in large scale, and locally high concentrations may inhibit or inactivate the enzymes.

However, the aim of this chapter is to provide a compressive review of the application potential of laccases in lignocellulose processing, such as delignification and detoxification of biomass by removal of phenolic compounds from hydrolysates and highlighting future prospects of the laccases in biofuel production (Table 1).

2 Laccases (Lac)

Laccases (EC 1.10.3.2, benzenediol-oxygen oxidoreductases) are polyphenol oxidases that belong to the multicopper oxidase family and the superfamily of cupredoxins. Laccases (EC 1.10.3.2, benzenediol-oxygen oxidoreductases) are polyphenol oxidases that belong to the multicopper oxidase family and the superfamily of cupredoxins. Laccase catalyzes oxidization of various substrates like diphenols, methoxy-substituted monophenols, as well as aliphatic and aromatic amines (Kudanga and Le Roes-Hill 2014). The fact that these polyphenol oxidases use O_2 as the final electron acceptor rather than H_2O_2, differentiate them from other ligninolytic enzymes. Moreover, they are cofactor independent and produce water as a sole by-product; they are very attractive biocatalysts for a variety of industrial applications. Laccases incorporate three copper atoms: one paramagnetic type 1 copper (T1 Cu), this is responsible for their characteristic blue color, wherein the oxidation of the substrate proceeds, one type 2 copper (T2 Cu), and an antiferromagnetically coupled binuclear copper pair of type 3 coppers (T3 Cu) that conform a trinuclear cluster wherein molecular O_2 is decreased to two molecules of H_2O (Mate and Alcalde 2015). During substrate oxidation, laccases receive electrons at the T1 copper sites from electron-donating substrate and then transfer the electrons to the trinuclear center (T2/T3) (Shleev et al. 2005). The substrate specificity and catalytic efficiency of the enzymes depend on the redox potential of the T1 Cu center, which is used to categorize the enzymes as low (0.35–0.5 V) or medium to high (0.5–0.8 V) redox laccases (Gutierrez et al. 2009). The main structural determinant believed to cause changes in the redox potential is the presence of an axial ligand at the T1 Cu center. The low-redox potential laccase from ascomycete fungus *Melanocarpus albomyces* ($E^0 = 0.46$ V) has a Met residue as an axial ligand, while high-redox potential laccases from basidiomycete fungi *Trametes hirsuta* ($E^0 = 0.78$ V) and *Trametes versicolor* ($E^0 = 0.80$ V) have a Phe residue as an axial ligand (Frasconi et al. 2010). It has been proposed that such an axial ligand helps to stabilize the center, thereby lowering the redox potential. If the redox potential of the phenolic substrate is higher than that of the enzyme, it needs a small compound called a mediator to be able to abstract an electron (see next section). Thus, to evaluate potential applications of a laccase, it is important to know its redox potential.

Regardless of an increase in the number of reports exploring the enzymatic activities of laccases, the role of laccases in lignocellulose or lignin processing remains unclear. Earlier studies showed the prominent involvement of laccases in lignin synthesis, and the consensus was that laccases do not take part in lignin degradation (Lundell et al. 2010). This is supported by studies using *P. chrysosporium*, which can degrade lignin but lack laccase activity. However, on the contrary, Eggert et al. (1997) identified laccase as the only ligninolytic enzyme predominantly secreted by the lignin-degrading fungi *Pycnoporus cinnabarinus*. Moreover, Xie et al. (2014) and Ryu et al. (2013) provided evidence toward the involvement of laccase in several stages of wood degradation by applying systematic gene deletion in the filamentous fungus *Podospora anserina* and overexpression of laccase in *Polyporus brumalis*. Also, Ander and Eriksson (1976) have shown possible involvement of laccase in lignin degradation by demonstrating that a laccase-deficient *Sporotrichum pulverulentum* mutant (obtained by UV mutagenesis) did not change lignin polymer; however, the wild type transforms the polymer easily.

3 Lignin

Lignin is a very composite biopolymer within the plant cell wall and is usually taken into consideration as a contaminant or glue in industrial applications which includes pulp/paper and biofuel production (Ko et al. 2015). It comprises approximately 20–30% of the lignocellulose, wherein it forms a matrix in close association with the cellulose and hemicellulose (Bugg et al. 2011).The lignin content varies between different types of plants, e.g., softwood contains 30%, hardwood 20–25%, and grass lignin only 10–15% of the total plant biomass. Due to the distinctly high-lignin content material in biomass, lignin removal is a crucial technical issue both for paper and bioethanol production (Chen and Dixon 2007). However, the lignin polymer is highly resistant to breakdown, and it is, therefore, considerable interest in methods to break down the lignin. Much attention has been drawn to the improvement of eco-friendly technologies for treating lignin with the aid of ligninolytic enzymes. Even though a number of the study's results are encouraging, there is still a far way to go, and numerous hurdles need to be addressed.

Structurally lignin is an amorphous tridimensional polymer of three different cinnamyl alcohol monomers: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Fig. 1a–c). The three monolignols differ in the degree of methoxylation and are catalyzed by an oxidative enzyme (e.g., peroxidases or laccases) to form the corresponding p-hydroxyphenol (H), guaiacyl (G), and syringyl (S) lignin components, respectively. As part of the increased interest in the utilization of lignin, a lot of effort has been put into studies of lignin structure and mechanisms involved in the synthesis. The three monomeric precursors are predominately linked either by ether or C–C bonds. In native lignin, two-thirds or more of the total linkages are ether bonds, and the rest are C–C bonds. During the radical polymerization process, the

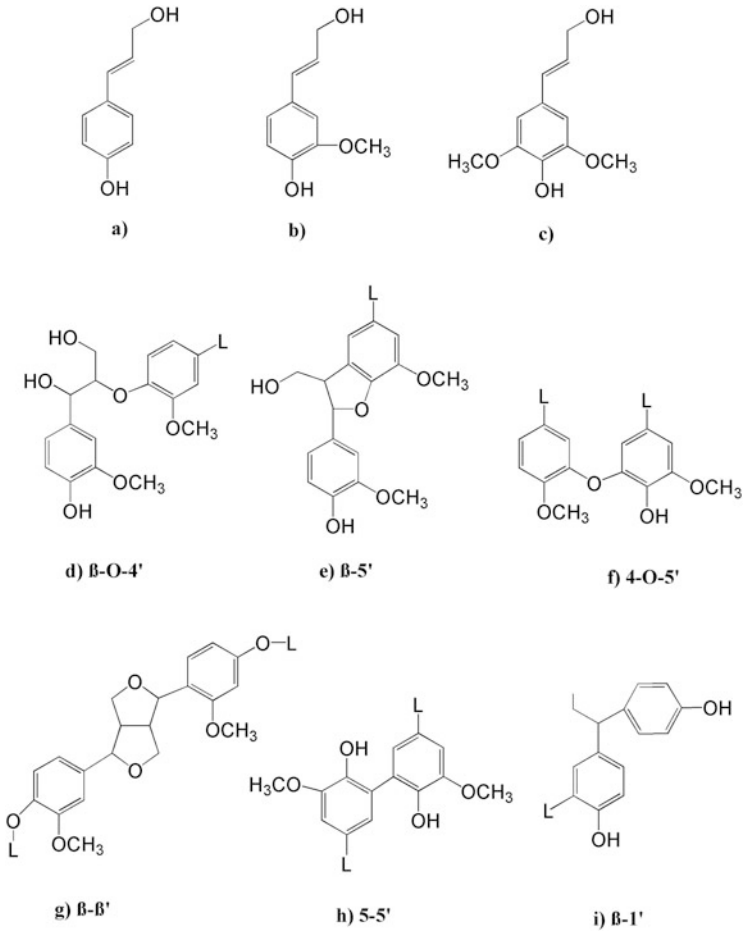

Fig. 1 The three major lignin precursors (**a**) p-coumaryl alcohol, (**b**) coniferyl alcohol, and (**c**) sinapyl alcohol and common linkages found in lignin (**d–i**)

complexity of lignin rises due to resonance delocalization of electrons, forming a three-dimensional, irregular matrix (Vanholme et al. 2010; Wong 2009). This results in a range of different ether and C–C linkages between the three major precursors, including β-O-4, β-β, α-O-4, 4-O-5, 5–5, α-O-γ, and β-1 (Li et al. 2015) (Fig. 1d–i). The binding types are, to some extent, affected by the content of the H-, G-, and S-units in the lignin (Djikanovic et al. 2012). Softwood lignins are often made out of G-units and minor quantities of H-units, whereas hard wood lignins are made out of G- and S-units in roughly equal ratios (Espineira et al. 2011). Softwoods are known to have a more branched structure compared to hardwood, which demonstrates a linear structure due to the higher amounts of S-units. Grass lignins contain significant amounts of G-, S-, and H-units, but the ratios between the three units and the resulting types of bindings vary within this group of plants (Buranov and Mazza 2008). The

composition of natural lignin is influenced by the plant's exposure to stress during synthesis (Moura et al. 2010), and it differs among cell kinds and may even range at the extent of individual cell wall layers (Vanholme et al. 2010). Moreover, lignin is covalently bound to the carbohydrate networks in the plant, which altogether impede a consistent determination of native lignin structures. Besides the variation in native structure, lignin-containing biomasses are exposed to different industrial processing which often implies the structural modification of the lignin (Zakzeski et al. 2010). Lignin-containing biomasses with native or industrially modified structures are frequently used in scientific studies to explore new and more sustainable conversion methods and applications (Munk et al. 2015).

The synthetic low molecular weight compound known as lignin model substrate has 1–3 aromatic rings that represent substructures and linkages similar to those observed in natural lignin (Rochefort et al. 2004). The most important compounds are those with β-aryl ether linkages (β-O-4) as they represent the majority of lignin bounds (Majumdar et al. 2014). Aromatic alcohols have also been used as model compounds. Phenolic subunits affect the redox potential (Polak and Jarosz-Wilkolazka 2012), which is closely related to the ability of the enzyme to catalyze oxidation reactions of the substrate. In native lignin, nonphenolic subunits comprise 80–90%, while the remaining 10–20% is phenolic subunits. A distinction between phenolic and nonphenolic lignin model compounds is therefore often used in studies of enzymatic lignin modification.

4 Laccase-Mediator System (LMS)

Mediators are small molecules (synthetic or natural) that help to carry electron in between laccase and normal laccase substrate (higher-redox potential than laccase) consisting of lignin. Overall, the laccase-mediator system helps to oxidize compound by relocating electrons from its phenoxy sites to oxygen. In this way, the mediator expands the oxidation ability of the enzyme. The function of mediators in laccase oxidations is outlined in Fig. 2a. The primary synthetic mediator to be used within the laccase-mediator approach for pulp oxidation/modification was ABTS (2,2′-Azino-bis(three-ethylbenzothiazoline-6-sulfonic acid), which used to be introduced in 1990 (Bourbonnais et al. 1995). They followed that laccase used in combination with a mediator (ABTS) was capable of extending the oxidation of lignin. Considering this discovery, numerous different compounds were delivered for use in the LMS centered for nonphenolic lignin oxidation. Among those are the –NOH mediators (1-hydroxybenzotriazole (HBT), *N*-hydroxyphthalimide (HPI), violuric acid (VLA), and *N*-hydroxyacetanilide (NHA)) (Shumakovich et al. 2006; Couto and Sanromán 2007; Moldes and Vidal 2008; Fillat et al. 2010).

The more effective oxidation and a broader range of substrates make the laccase-mediator system interesting for industrial application. It has been verified that depending on their chemical structure, mediators follow three different kinds of

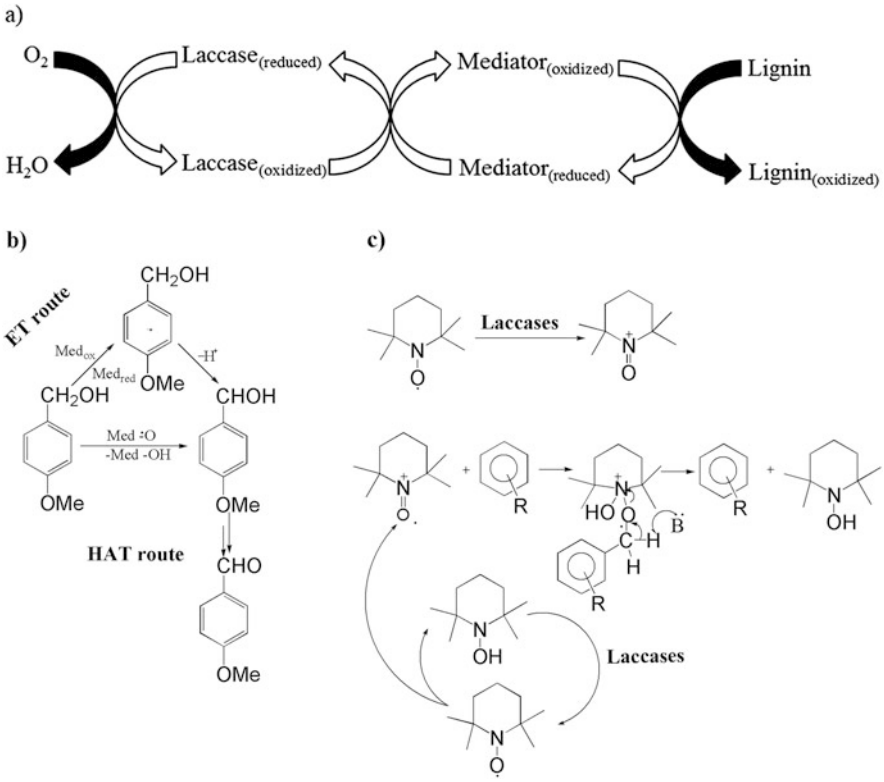

Fig. 2 (**a**) The overall oxidation and reduction reactions of the laccase and LMS to delignification. (**b**) The ET and radical HAT route, (**c**) suggested mechanism of oxidation by TEMPO (Astolfi et al. 2005)

oxidation mechanism: (I) electron transfer (ET) in the case of ABTS radicals ($ABTS^{\bullet+}$or $ABTS^{2+}$), where the mediator abstracts an electron from the substrate.

The particular generating pressure intended for electron abstraction is the distinction in redox potential between the mediator and substrate (Fig. 2b). (II) Hydrogen atom transfer (HAT) for nitroxyl radicals (N–O$^{\bullet}$) 1-hydroxybenzotriazole (HBT) and violuric acid (VLA). The oxidation of mediators through laccase generates an especially reactive radical (> N–O$^{\bullet}$), because of the enzymatic elimination of an electron followed by the release of a proton (Fig. 2b). (III).The ionic mechanism is observed for mediators such as TEMPO (Astolfi et al. 2005) (Fig. 2c). LMS were utilized to numerous procedures and exhaustively reviewed several occasions in contemporary years (Riva 2006; Morozova et al. 2007; Husain and Husain 2008; Kunamneni et al. 2008; Widsten and Kandelbauer 2008; Canas and Camarero 2010; Kudanga et al. 2011; Christopher et al. 2014; Kudanga and Le Roes-Hill 2014; Forootanfar and Faramarzi 2015; Munk et al. 2015; Roth and Spiess 2015; Singh et al. 2015). From the documented studies and reviews, it is possible to outline or

define the required characteristics for effective mediators. A mediator must be a good laccase substrate, implying that its oxidized and reduced forms must be steady without inactivating the enzymatic reaction and that its reactivity must permit recycling without degeneration.

From an industrial application and environmental point of view, laccase mediators must be eco-friendly and cost-effective. Despite promising results associated with synthetic mediators in LMS (Martin-Sampedro et al. 2011; Gutierrez et al. 2012), there are few drawbacks interfering with the effective use of mediators: they are expensive and they can create toxic compounds. Furthermore, in some reaction, even as oxidizing the mediator, the laccases are deactivated via radicals, or the mediators may be loss mediating capability by transforming into inactive compounds (Kunamneni et al. 2008). Some of these problems could be solved by utilizing the natural mediators and have come around with an increasing interest in the recent years. The important advantage of utilizing natural phenols as mediators is that they are easily obtained from plant materials. Additionally, their low price and toxicity can offer economic and environmental benefits (Canas and Camarero 2010). Lignin-derived phenols used as laccase mediators have been found to perform in a similar way to or even better than the artificial mediator compounds, with elevated reactivity (Fillat et al. 2010).

5 Laccases in Lignocellulose Processing

Laccases have recently attracted attention as candidate enzymes for the biological pretreatment of biomass. Due to their likely role as lignin-degrading enzymes, they offer the potential for a highly specific and environmentally friendly method for removing lignin.

Improved knowledge of laccases/laccase-mediator system and their role in lignin degradation will have a significant impact on a wide range of applications focused on lignin degradation. Even though the effect of laccases on lignin has been intensively studied, the only consensus in the literature is that laccases oxidize subunits of lignin into reactive radical intermediates, which lead to lignin modifications.

Earlier studies have investigated the reactivity of laccases by using lignin model compounds. Both phenolic and nonphenolic dimeric β-O-4 lignin model compounds (as more than 50% of lignin structure is composed of β-O-4 bonds) are widely used to study Cα-Cβ bond cleavage by the formation of phenoxy radicals. This leads to α-oxidations, C_α-C_β, alkyl-aryl, or alkyl-phenyl cleavages that form a range of products (Higuchi 2004; Wong 2009). The phenolic monomers present on the surface of the lignin polymer can be easily oxidized by laccase alone. In studies related to the role of LMS in degradation of lignin or lignin model compounds, it has been found that mediators facilitate a strong lignin oxidation capability in combination with laccases. Several of these studies show a decrease in lignin molecular weight suggesting depolymerization (Martin-Sampedro et al. 2011; Zheng et al.

2012; Rico et al. 2015). However, both depolymerization and polymerization reactions may take place depending on whether the treatment is with laccases alone or with a laccase-mediator system (Shleev et al. 2005). A range of studies where laccases were used in the presence of synthetic mediators like HBT and proven to effectively depolymerize different forms of biomasses(Chen et al. 2012; Gutierrez et al. 2012; Rico et al. 2014). The elucidation of the recently discovered LMS (*Pycnoporus cinnabarinus* laccase-HBT) has extended the substrate activity of laccase to include oxidation of nonphenolic lignin model compounds (Du et al. 2013). Figure 3 illustrates the oxidation mechanism of nonphenolic substrate (β-O-4 compound). During the oxidation process, LMS is able to form carbon-centered radicals by oxidation of both S- and G-units of nonphenolic lignin units (structure I). In the next step, O_2 attacks the carbon-centered radical intermediate, forming unstable structure (structure IIa, β-aryl, or benzylic radical) in a nonenzymatic reaction. The consecutive reactions lead to additional Cα oxidation (structure III), β-ether cleavage, and aromatic ring cleavage. The alternative route involves suggesting radical structure IIa abstract proton from a substrate to form nonradical structure IIb. This can be followed by the cleavage between Cα and Cβ forming structures IV and V (Kawai et al. 2002; Du et al. 2013). Recently, Rico et al. (2014, 2015) showed a decrease in lignin molecular weight with shortened side chains and increased syringyl-to-guaiacyl ratio by applying LMS with laccase from *Myceliophthora thermophila* and natural mediator methyl syringate. Contradictory, application of LMS has also shown that the oxidizing capability of some mediators leads to polymerization of lignin (Moya et al. 2011), and this has also been demonstrated for ABTS and HBT (Prasetyo et al. 2012).

The choice of mediator is an important factor for boosting the effectivity of laccases. Beside mediators, several other factors such as biomass processing, reaction condition in the form of pH, type of solvent, temperature, and other small compounds present may influence the reaction outcome. Shleev et al. (2006) observed different molecular weight in oxidative compounds in response to two of most commonly applied mediators, ABTS and HBT, which support the fact that these two mediators act through different mechanisms. This finding is supported by another study by Hernández Fernaud et al. (2006), who reported similar results using the same mediators but different laccase and substrate. Thus, the type of mediators seems to be important for which reactions laccases may catalyze. However, this topic is rarely addressed in the literature (Moldes and Vidal 2012). In spite of the enormous information availability on the laccase reaction with lignin model compounds and different varieties of natural lignins, a comprehensive knowledge of the LMS is still lacking. With an appropriate mediator at hand, the LMS be examined both for an evaluation of the overall performance of synthetic and natural mediator or to clarify their mechanism of action. Nevertheless, it is also important to use real lignin polymers in experiments. It is also important to be aware of the presence of possible natural mediators in the substrates, which may also influence the balance between polymerization and depolymerization.

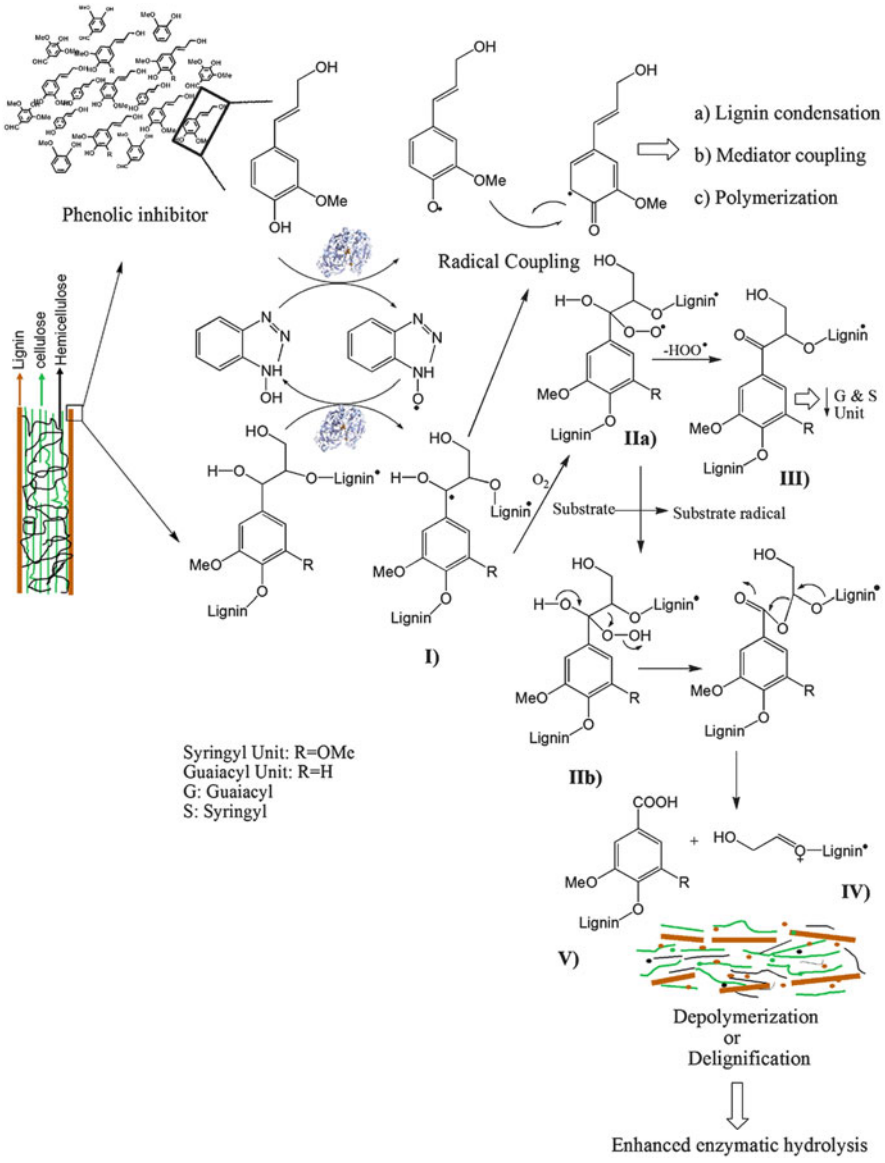

Fig. 3 Systematic delignification mechanism of the lignocellulose substrate by laccase or laccase-mediator system. The LMS-catalyzed nonphenolic oxidation (β-O-4) occurs in four types of reactions such as Cα-oxidation reactions (structure III), followed by β-ether cleavage and aromatic ring cleavage, further Cα-Cβ cleavage (structure IV and V) led decrease in lignin molecular weight (Kawai et al. 2002; Du et al. 2013; Kudanga and Roes-Hill 2014). However, in case of phenolic monomers, laccases subtract electron from –OH of phenolic monomer to form phenoxy radicals; further it undergoes polymerization or condensation via radical coupling (Kudanga and Roes-Hill 2014)

The cost of enzyme is one of the fundamental challenges in present-day large-scale biofuel production process. However, several strategies for cost reduction are investigated. For instance, enzyme co-expression or chimeras between laccases and cellulolytic enzymes could help to reduce cost and restrictions of conventional workflow for biofuel production from lignocellulosic biomass (Fonseca-Maldonado et al. 2014). The co-expression of laccases with xylanases or endoglucanases helps to improve catalytic activity, resulting in higher glucose yields (Ribeiro et al. 2011; Furtado et al. 2013; Fonseca-Maldonado et al. 2014). Recently, Zhao et al. (2016) have combined the laccase and Fenton reaction system to assessing the synergism in lignin depolymerization. Apparently, bacterial fermentation (*Rhodococcus opacus*) in the presence of laccase led to significant improvements in hydroxyl group degradation, lignin molecular weight, cell growth, and overall higher lignin consummation and improved lipid production (17-fold). However, immobilization of laccase on Sepa beads carriers has been shown to polymerize toxic phenolics from hydrolysate by precipitation onto the carrier surface. Incorporation of anion exchange as a subsequent step led to the reduction of HMF, formic acid, acetic acid, and levulinic acid and helped to improve the ferment ability of an organosolv wheat straw hydrolysate (Ludwig et al. 2013). Immobilization of enzymes is advantageous for commercial application due to convenience in handling and recycling and improves stability. These capabilities can be exploited for designing eco-friendly conversion of lignin toward numerous useful products or third generation of biorefineries.

Enzymatic/LMS delignification followed by alkali pretreatments can increase the enzymatic hydrolysis yields despite the fact that removal of lignin does not drastically improve (Gutierrez et al. 2012; Li et al. 2012). However, Li et al. (2012) reported combination of alkaline and laccase treatment significantly increases porosity and surface area of corn straw, which result in noteworthy saccharification yield as compared to alkaline treatment alone. Gutiérrez et al. (2012) observed the same effect for wood (*Eucalyptus globulus*) and non-wood (*Pennisetum purpureum*) biomass using a *Trametes villosa* laccase, in combination with alkaline extraction and HBT.

6 Techniques for Identifying Enzymatic Lignin Degradation

The use of established tools and techniques and the improvement of novel assays are essential to progress research on lignocellulosic biofuels. Such tools and techniques additionally inform us on the structure and conformation of lignin and enable us to assess the various strategies for biomass processing. To evaluate or elucidate the mechanism of biological degradation or modification of the lignin is a challenging task, because of its complex macromolecular structure and intimate association to cellulose and hemicellulose (Gellerstedt and Henriksson 2008).

Analytical methods exist as either nondestructive (mostly spectroscopic) or destructive (use chemical or thermal degradation and fragment analysis) techniques. Nonetheless, some cutting-edge analytical techniques such as two-dimensional (2D) or three-dimensional (3D) nuclear magnetic resonance (NMR) can be used for the evaluation of complicated macromolecules like lignin (Gutierrez et al. 2012; Nugroho Prasetyo et al. 2010; Salanti et al. 2010; Rico et al. 2015). The combination of quantitative 2D or 3D NMR with ^{1}H and ^{13}C NMR spectroscopic techniques represents an extraordinary advance in the structural analysis of this complex polymer (Balakshin et al. 2011; Liu et al. 2014; Mori et al. 2015; Rico et al. 2015). Moreover, fluorescence monitoring, Fourier transform-infrared (FTIR) spectroscopy, and size exclusion chromatography (SEC) are also used to study chemical changes in lignin (Ibarra et al. 2007; Maijala et al. 2012; Sun et al. 2013; Majumdar et al. 2014; Oliva-Taravilla et al. 2015; Rajak and Banerjee 2015). The usage of pyrolysis technique in combination with mass spectrometry (Py-GC/MS) has proved to be of specific interest within the look at of lignocellulosic macromolecules (Rio et al. 2002; Dey Laskar et al. 2013; Du et al. 2013; Heap et al. 2014).

7 Conclusion

Laccases are potent enzymes for industrial applications in sustainable biomass conversion, but some hurdles need to be overcome before this can be realized. In spite of intensive studies in the field, it is still not clear how to control and most efficiently utilize the laccases. The type of mediator and biomass appear to influence the resulting lignin oxidation, but it is likely that the importance of other factors may be discovered. Accordingly, laccase-catalyzed detoxification or depolymerization needs to be properly incorporated into relevant process steps of the biorefinery. By exploring greater diversity of the laccase-producing organisms (which can react with and metabolize lignin) and discovering new natural mediators (which can be applied in an LMS system for lignin depolymerization), we may be able to gain new insight into strategies for biomass deconstruction. A more thorough interpretation of analysis methodologies along with application of representative reference enzymes, mediators, and model substrates may facilitate the increased understanding of the mechanisms that occur when laccases interact with lignocellulosic biomass.

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