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Environmental Microbiology and Biotechnology

Volume 2: Bioenergy and Environmental Health

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Anoop Singh • Shaili Srivastava •
Dheeraj Rathore • Deepak Pant
Editors

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Foreword

Increasing human population and developing civilizational goals in terms of quality of living and subsequent energy needs have led to extreme pressures on the finite resources that the earth has to offer. Especially the conventional energy derived through petroleum resources and coal reserves is rapidly declining, leading to concerns as well as global interest in developing alternative sources of energy. At the same time, environmental health is also declining due to several anthropogenic activities. It is therefore important to develop tools and technologies to measure and monitor the quality of the environment in order to find approaches for remediating the contaminated environments as well getting useful products out of this process in the current vogue approach of resource recovery. In the current environmentally and sustainably conscious times, the concept of waste has been replaced with resource.

Microbiology and biotechnology have traditionally found application in both these aspects of bioenergy production, bioremediation and extracting useful products from otherwise hitherto waste materials. Governments and industries all over the world are considering the potential of microorganisms and biotechnology to develop biomass-based processes for the development of liquid and gaseous fuels, as well as algal fuels, biogas upgrading, aviation biofuels, bioelectrochemical systems all of which have been covered extensively in this book. Lignin depolymerization aquatic microbial oxygenic phototrophs offer much promise and are discussed in detail.

In addition to the role of microbial biotechnology in creating an alternative source of energy, the book also takes a good look at the environmental health issues.

Bioinformatics as a tool to deal with environmental management is elaborated. Non-traditional pollutants such as pharmaceuticals and dioxins are described in detail and so are their impact on stem cells and human health.

The book on 'Environmental Microbiology and Biotechnology' presented in two volumes provides state-of-the-art information on different aspects of microbes and their application in areas as diverse as solid waste management, sustainable agriculture and wastewater treatment. This particular volume deals with bioenergy and environmental health aspects. The editors have put together a host of highly relevant topics and experts in their respective fields to contribute thoroughly the described chapters.

Overall, the information provided in this book is fully consistent with the current state of the art with practical information and would be beneficial for the researchers and practitioners equally beside the young researchers who are entering into this highly relevant and important area.

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Preface

The ever-growing energy demand and environmental pollution have induced the search for technologies to harness the power curbed in the biomass and/or biowaste. This will not only provide the energy but also resolve the disposal burden and environmental pollution due to faulty disposal of biowaste. The microbiological and biotechnological techniques can play a great role in finding green and sustainable solutions to the problems related to energy and environment. Environmental health is broadly concerned with assessing, understanding and controlling the impacts of anthropogenic activities on the environment and the impact of environment on people. A number of environmental health problems, like effects of toxic chemicals, resource depletion, etc. have been analysed by researchers. The various microbial strains can be employed for solving different problems of environmental health such as degradation of toxic chemicals and production of value-added products from them, restoring the resources, synthesis of beneficial compounds, etc. Biotechnological tools give an added advantage over microbiological techniques by enhancing the efficiency of employed microbes.

The present book is the second volume of the book 'Environmental Microbiology and Biotechnology'. In the first volume, we covered three parts, namely: I. solid waste management, II. agriculture utilization and III. water treatment technology. This volume covers two sections, namely: IV. bioenergy and V. environmental health. In Part IV, various biotechnological and microbiological approaches are described for sustainable energy production such as algal biofuel, biogas production, aviation biofuels, etc. This part also includes microbiology of bioelectrochemical system and applications of biotechnology in oil and gas industries. Part V extends the discussion about applications of microbial biotechnology for better environmental health and covers the topics related to bioinformatics tools for hazardous waste management, modern biotechnological tools for pathogen analysis, environmental interaction of stem cells, pharmaceutical waste management, pathogens, genetic variations and cancer, CO_2 sequestration by microbiological approach, metabolism of dioxins, remediation of heavy metals degradation of phenols, food safety and preservation, biotransformation of xenobiotics compound, etc.

The present book is a comprehensive collection of chapters on the utilization of microbiological and biotechnological tools for energy production and improvement of environmental health.

The book is aimed at a broad audience, mainly researchers, environment specialists, academicians, entrepreneurs, industrialists, policymakers and others who wish to know the latest development and future perspectives of microbial and biotechnological approaches for bioenergy (biogas and biodiesel) production and upliftment of the environment. The book is written in a lucid style and comprehensively covers each point to give the reader a holistic picture about environmental microbiology and biotechnology and its future perspective for bioenergy production and benefits of environmental health. The book may even be adopted as a textbook for masters and undergraduate university courses that deal with environmental microbiology and biotechnology.

Despite the best efforts of authors and editors along with extensive checks conducted by many experts in the field of environmental microbiology and biotechnology, mistakes might have crept in inadvertently. We would appreciate if the readers could highlight these and make comments or suggestions to improve and update the book contents for future editions.

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Part IV

Bioenergy

1 Algal Biofuel: A Sustainable Approach for Fuel of Future Generation

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Abstract

Fossil fuels are used for the generation of electrical power, as well as liquid fuels. There are a variety of renewable or low atmospheric pollution technologies that can generate electrical power, including solar, wind, hydroelectric, geothermal, and nuclear. However, renewable technologies to supplement or replace liquid fossil fuels are still in their early developmental stages. The International Energy Agency expects that biofuels will contribute 6% of total fuel use by 2030, but could expand significantly if undeveloped petroleum fields are not accessed or if substantial new fields are not identified. The use of liquid fossil fuel as an energy source has long been considered unsustainable and most importantly the liquid fossil fuel will be diminished by the middle of this century. In addition, the fossil fuel is directly related to environmental degradation and greenhouse emission. Biofuel produced from plants, animals, or algae products can offer an alternative to reduce our dependency on fossil fuel and assist to maintain healthy global environment. Microalgae are becoming popular candidate for biofuel production due to their high lipid contents, ease of cultivation, and rapid growth rate. Fatty acid methyl esters (FAME) are used as alternative diesel fuel originating from renewable sources. The attention is focused on the materials that do not compete with food and feed production. Algae have a significant potential as an alternative biodiesel feedstock. In comparison to other crops, they have the advantage in very fast reproduction cycles, enhanced resistance to high UV radiation doses, and higher effectiveness of energy conversion to biomass due to low demands on other metabolic functions. Moreover, they fix atmospheric CO_2 very efficiently. Microalgae have benefit over other sources of biofuel as it fixes atmospheric CO_2

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very efficiently and also fulfill liquid fuel demand for present as well as future generation.

1.1 Introduction

The demand for liquid fuels in transport is rising. Nowadays, fatty acid methyl esters (FAME) are accepted liquid biofuels for diesel engines. They are usually prepared from vegetable oils or animal fats. Today, 90% of organic chemicals are based on fossil fuels, meaning they are based on non-renewable resources. Seventy percent of proteins in the European Union are imported. An alternative is needed. Due to their high growth rate and the small land area required, algae could become that alternative. We are not at that stage just yet. The challenge is to optimize the algae value chain, from local production to storage and handling, in such a way that it becomes a viable, economically relevant industry. Worldwide, algal biofuel research and development efforts have focused on increasing the competitiveness of algal biofuels by increasing the energy and financial return on investments, reducing water intensity and resource requirements, and increasing algal productivity (Herzog et al. 2001).

1.1.1 Interest in Sustainable Development of Algal Biofuel

The aspiration for producing algal biofuel is motivated by the desire to: (1) displace conventional petroleum-based fuels, which are exhaustible, (2) produce fuels domestically to reduce energy imports, and (3) reduce greenhouse gas emissions by cultivating algae that reuse carbon dioxide emitted from industrial facilities. In theory, algae have the potential to produce a large amount of petroleum fuel substitutes while avoiding the need for large amounts of fresh water and arable land. These attributes have created widespread interest in algal biofuels. In practice, however, profitable algal biofuel production faces several important challenges (Slade and Bauen 2013).

Microalgae are a large and diverse group of aquatic organisms that lack the complex cell structures found in higher plants. They can be found in diverse environments, some species thriving in freshwater, others in saline conditions and seawater. Most species are photoautotrophic, converting solar energy into chemical forms through photosynthesis (Elegbedea et al. 2017). Microalgae have received considerable interest as a potential feedstock for biofuel production because, depending on the species and cultivation conditions, they can produce useful quantities of polysaccharides (sugars) and triacylglycerides (fats). These are the raw materials for producing bioethanol and biodiesel transport fuels. Microalgae also produce proteins that could be used as a source of animal feed, and some species can produce commercially valuable compounds such as pigments and pharmaceuticals (Chisti 2007). There are two main alternatives for cultivating

photoautotrophic algae: raceway pond systems and photobioreactors (PBRs). A typical raceway pond comprises a closed loop oval channel, w0.25e0.4 m deep, opens to the air, and mixed with a paddle wheel to circulate the water and prevent sedimentation (Ponds are kept shallow as optical absorption and self-shading by the algal cells limit light penetration through the algal broth). In PBRs the culture medium is enclosed in a transparent array of tubes or plates and the microalgal broth is circulated from a central reservoir. PBR systems allow for better control of the algae culture environment but tend to be more expensive than raceway ponds. Auxiliary energy demand may also be higher. The perceived potential of microalgae as a source of environmentally sustainable transport fuel is a strong driver behind their development and provides the underpinning rationale for much of the public support directed towards microalgae R&D. It is important, therefore, that algae biofuel systems are able to clearly demonstrate their environmental and longer-term economic credentials. Researcher explained three aspects of microalgae production that will ultimately determine the future economic viability and environmental sustainability: the energy and carbon balance, environmental impacts, and production cost (Hannon et al. 2010a, b).

1.2 Algae: Promising Future Feedstock for Biofuels

Microalgae are currently being promoted as an ideal third generation biofuel feedstock because of their rapid growth rate, greenhouse gas fixation ability (net zero emission balance), and high production capacity of lipids (fat). They also do not compete with food or feed crops, and can be grown on non-arable land and saline water. Biofuels are generally referred to solid, liquid, or gaseous fuels derived from organic matter (Chisti 2007). The classification of biofuels is shown in Fig. 1.1.

The oil contents of various microalgae in relation to their dry weight are shown in Table 1.1. It is clear that several species of microalgae can have oil contents up to 80% of their dry body weight. Some microalgae can double their biomasses within 24 h and the shortest doubling time during their growth is around 3.5 h which makes microalgae an ideal renewable source for biofuel production.

Oil productivity, the mass of oil produced per unit volume of the microalgae broth per day, depends on the algal growth rate and the oil content of the biomass. The yield of the oil produced by algae is significantly higher (100,000 L/ha) in comparison to other crops, for example, soybean (446 L/ha), sunflower (952 L/ha), rapeseed (1200 L/ha), castor (1413 L/ha), coconut (2689 L/ha), and palm (5950 L/ha). There exist three distinct algae production mechanisms including photoautotrophic, heterotrophic, and mixotrophic production. Currently, photoautotrophic production is the only method which is technically and economically feasible for large-scale production of algae biomass for non-energy production. Two developed systems are based on open pond and closed pond photobioreactor technologies. High algae production rates are achievable with open pond systems. The recovery of microalgal biomass which generally requires one or more solid–liquid separation steps is a challenging phase of the algal biomass process, and accounts for 20–30% of the total

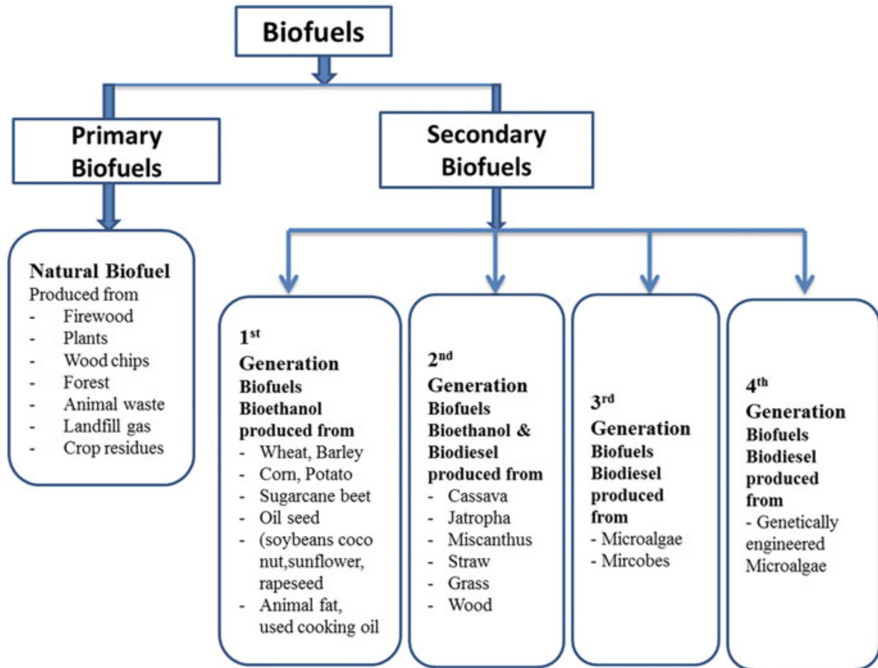

Fig. 1.1 The classification of biofuels (adapted from Dragone et al. 2010)

costs of production. The selection of harvesting technology is crucial to economic production of algal biomass (Zhou et al. 2014). The processes involved include flocculation, filtration, flotation, centrifugal sedimentation, also extraction and purification. Well-known methods of the oil extraction are: (1) Expeller/Press, (2) solvent extraction, and (3) supercritical fluid extraction. The most popular chemical solvent for extraction is hexane. Supercritical fluids are selective, thus providing the high purity and product concentration. This can extract almost 100% of the oils all by itself (Elegbedea et al. 2017).

1.3 Process of Algal Biomass Into Biofuels

1.3.1 Cultivation of Algae

Cultivation of microalgae can be conducted in batch, semi-batch, and continuous systems. Batch culture consists of a single inoculation of cells in container of media over several days of growth period until the cell density reaches a maximum/ desirable level ready to be transferred to larger culture volumes to continue growth before reaching the stationary phase. The semi-batch system allows a portion of the culture to be harvested and replenished with fresh medium. In a continuous system,

Table 1.1 Oil content of microalgae (Sajjadi et al. 2018)

S. No.	Species	Oil content	References
1	*Dunaliella primolecta*	23%	Sajjadi et al. (2018)
2	*Dunaliella salina*	6–25%	Sajjadi et al. (2018)
3	*Nannochloris* sp.	25-56%	Vasudevan and Briggs (2008)
4	*Chaetoceros muelleri*	13–24%	Rodríguez et al. (2012)
5	*Scenedesmus obliquus*	30–50%	Caprio et al. (2015)
6	*Chlamydomonas* sp.	22.7%	Jeon et al. (2015)
7	*Parietochloris incisa*	62%	Sajjadi et al. (2018)
8	*Tetraselmis tetrathele*	25–30%	Sajjadi et al. (2018)
9	*Neochloris oleoabundans*	35–65%	Vasudevan and Briggs (2008)
10	*Chlorella* sp.	28–53%	Vasudevan and Briggs (2008)
11	*Nostoc commune*	22%	Sajjadi et al. (2018)
12	*Spirulina Platensis*	4–11%	Sajjadi et al. (2018)
13	*Synechocystis* sp.	11%	Abdelkhaalek et al. (2016)
14	*Emiliania huxleyi*	43.8%	Sajjadi et al. (2018)
15	*Heterosigma akashiwo*	43%	Stewart (2014)
16	*Chroomonas salina*	12–14.5%	Sajjadi et al. (2018)
17	*Porphyridium cruentum*	9–14%	Sajjadi et al. (2018)
18	*Mesotaenium* sp.	19–35%	Sajjadi et al. (2018)
19	*Schizochytrium* sp.	50–77%	Sun et al. (2014)

two types of culture can be used: turbidostat and chemostat culture (Ghasemi et al. 2012). In the turbidostat culture, when the density reaches a preset level, fresh medium is added to the culture as the cells continue to divide and grow. In the chemostat culture, a slow but steady flow of fresh medium is continually introduced into the culture while excess culture overflows and collected. Two types of reactors have been developed to cultivate algae: open system (such as raceway ponds) and closed system (such as photobioreactors) (Brennan and Owende 2010; Ghasemi et al. 2012; Narala et al. 2016).

1.3.1.1 Open Pond Systems

Open pond system is the oldest system and can be categorized into natural and artificial water ponds or containers (Jimenez et al. 2003). Raceway ponds are the most commonly used artificial system for mass cultivation of microalgae. It is usually 0.2 and 0.5 m deep in length to thousands of meters and consists of a pond in the shape of a raceway. The raceway ponds mimics the way of algae to grow in their natural environment in which the liquid is circulated around the pond by a paddle wheel to stabilize algae growth and productivity. Raceway ponds are usually made from poured concrete and compacted into the earth with white plastic liner. Due to scalability and low cost of building, they are the most popular artificial cultivation system (Singh et al. 2011). In open pond systems, algal medium and their nutrients are introduced in front of the paddle wheel and circulated using the loop for

continuous production cycles (Terry and Raymond 1985). Circular ponds, another type of open pond system, are also used for algal cultivation (Benemann and Oswald 1996). This is more popular for wastewater treatment by using algal culture. These ponds system contain a centrally located rotating arm to mix the algae culture. Raceway and circular ponds are mixed open pond systems that keep algae suspended culture. Open pond systems are also included some unmixed open ponds for algal culture. Unmixed ponds allow the algae to settle to the bottom of the reactor and usually used to grow specific species of algae with very low productivities (Borowitzka 1999). Open ponds systems are cheaper than other systems in use for large-scale algal production (Chisti 2008). These are not competing for land with existing agricultural crops and can be implemented in areas with marginal crop production potential. They also require lower energy input requirement. Open ponds systems are subjected with regular maintenance and cleaning and have the potential to give large net energy production. In 2008, open pond system was resulted about €2.55/kg of dry biomass of commonly cultivated algae strain, *Dunaliella salina*, which was considered to be too high for biofuel production.

Major Issues of Open Pond Systems In open ponds, major issue is the temperature control that usually fluctuates in a diurnal cycle and very difficult to control due to seasonal based fluctuation in a diurnal cycle (Mata et al. 2010). Other significant issue is evaporative water loss from open pond system due to which CO_2 utilization by algae is much less effective than other systems (Oyler 2009; Narala et al. 2016). This potential CO_2 deficiency may result in reduced biomass productivity. There is a poor mixing of algal culture through inefficient stirring that can result in poor mass CO_2 transfer rate (Chisti 2007). Minimum light intensity due to top layer thickness may also result in low algal biomass production. Due to significant contamination by native species, this system is subjected for only a small number of algae species that can tolerate extreme saline and alkaline environments of systems (Laws et al. 1988). For example, the species *Chlorella*, *D. salina* and *Spirulina* are adaptable to thrive under nutrient-rich, very high salinity, and high alkalinity condition of systems (Borowitzka 1999; Brennan and Owende 2010).

1.3.1.2 Closed Systems

The major issues associated with open pond cultures such as low cell densities, contamination issues, evaporation, environment regulation, and high land requirements have been overcome after the establishment of closed photobioreactors (PBRs) (Narala et al. 2016). Closed systems are facilitated by indoors with artificial light and outdoors with natural light. PBR's require higher capital investments and have issues with scalability. Unlike the open pond systems, closed systems allow the single algal species cultivation for long duration with low risk of contamination. The closed configuration of these systems makes the control of potential contamination easier. Closed systems are consisting of tubular, flat plate, and column photobioreactors type systems. Closed systems attain higher cell mass productivities and reduced harvesting costs. Photobioreactors are made up of an array of straight glass or plastic tubes (Narala et al. 2016).

Tubular PBR The most widely used PBR is of tubular design which is composed of an array of straight glass or plastic tubes (Chisti 2007). These tubes are generally 0.1 m or less in diameter and can be aligned horizontally, vertically, inclined, or as a helix. These tubes allow the sufficient sunlight penetration for photosynthesis. The culture is circulated through the tubes and then returns to a reservoir. Re-circulation of algal biomass as turbulent flows is maintained through airlift pump inside the reactor. Excess amount of dissolved O_2 in the medium is reduced through agitation and mixing by using gas exchange chambers.

Helical PBR Helical PBRs are made up of a cylinder and coiled parallel transparent tubes. Increased surface area of helical PBR is allowing sufficient sunlight to reach, thus increased productivity attained as compared to tubular PBR (Carvalho 2006). However, its unique shape and increased cost did not make it as popular.

Airlift PBR An airlift PBR is composed of a simple vertical cylinder made out of transparent glass or plastic. There is an air inlet on the bottom of the tube to create air bubbles through the column for sufficient mixing and gas exchange. These systems provide aerial productivity of algae as compared to tubular reactor.

Flat Panel (Flat Plate) PBR Flat panels, also known as flat plate PBRs, are consisting of rectangular boxes made up of transparent materials for maximum solar energy capture. A thin layer of algal culture flows across the flat plate. Air inlet on the bottom provides bubbles which provide sufficient mixing and gas transfer. It is the earliest form of closed system which has received research attention toward mass cultures of algae due to large surface area exposed to illumination and low accumulation of dissolved oxygen. Because of the increased surface area for light, higher algae productivity is achieved in flat plate PBR as compared to open pond system. For example, in one study of algae *S. platensis*; the flat panel PBR produced 2.15 g L^{-1} day^{-1} of algal biomass and an open pond produced 0.15 g L^{-1} day^{-1} of algal biomass (Carvalho 2006).

1.3.2 Harvesting Methods

1.3.2.1 Flocculation

Flocculation is the first stage of harvesting process that is intended to agglomeration of algae cells in order to increase the effective “particle” size. Flocculation is a preparatory step prior to other harvesting methods (filtration, flotation, or gravity sedimentation) (Golueke and Oswald 1965; Molina et al. 2003). The addition of flocculants (multivalent cations and cationic polymers) neutralizes the negative charge of microalgae cells and causes cells to form large clumps. These clumps can be easily fall out of suspension. Different methods of flocculation have been introduced for algal cell suspension.

Chemical Flocculation Inorganic molecules such as aluminum sulfate, ferric sulfide, or lime are suitable flocculants. These molecules can neutralize the cells charge or reduce it (Sheehan et al. 1998). However, the use of these chemicals leads to buildup of undesirable compounds in the harvested cells which leads to issues in using the algae as animal or human food. Instead of inorganic flocculants, the addition of highly charged organic molecules such as polyelectrolytes is more suitable to neutralize the algal cells. Polyelectrolytes can also physically link algal cells together, which helps in very stable flocs formation. They also do not have the toxicity that many of the inorganic molecules cause. This makes them a much more attractive chemical option for flocculation.

Electroflocculation In this method an electric current is passed through the culture to form flocs. This method is increasingly popular with very high separation efficiencies. It can also be applicable for large-scale cultures without utilizing a high amount of electricity.

Bioflocculation It is accomplished by stimulating limiting nitrogen or altering pH and dissolved O_2 levels. Addition of any external material or force to the culture is not required. Although this method does not need any additional cost for flocculants, it generally requires a longer time and is unreliable.

1.3.2.2 Flotation

Flotation method does not require any addition of chemicals. In this method, algal cells (flocs) are trapped at the surface by dispersed micro air bubbles in the liquid (Phoochinda and White 2003). Some strains can naturally float at the surface of the water as their lipid content increases.

1.3.2.3 Centrifugal Sedimentation

It is based on Stoke's law and is widely used in many liquid–solid separations. This technology utilizes centrifugal forces to separate substances of different densities. Separation efficiency is based on the density and radius of desired algal cells. Algal cells separation is based on algal cell density, residence time during centrifugation, and settling depth. This method is very effective for algal cells separation from liquid; however, it is not a feasible technique for large-scale algal culture due to high energy requirement and maintenance cost (Davis et al. 2012).

1.3.2.4 Filtration

Filtration is used to separate substance according to particle and screen pore size. Filtration can be an effective method for harvesting larger strains of algae. The algae *Spirulina* sp. can be simply filtered from the culture medium. Although filtration can be inexpensive and an effective method for harvesting algae, it has the major issue of filter fouling and clogging, which limit its application in large-scale cultivation.

1.3.3 Extraction of Algal Oil

The harvested biomass must be processed for extraction of desired final product. Methods that have been used include sun drying, low-pressure shelf drying, spray drying, drum drying, fluidized bed drying, freeze drying, and refractance Window TM technology drying (Molina et al. 1994; Prakash et al. 1997; Leach et al. 1998; Desmorieux and Decaen 2006; Nindo and Tang 2007). Sun drying is the cheapest dehydration method; but it takes long time, and required large drying surfaces with the risk of material loss (Prakash et al. 1997). Spray drying can be used for oil extraction but it is relatively expensive and can cause significant deterioration of some algal pigments commonly (Desmorieux and Decaen 2006). Freeze drying is also expensive, during large-scale operations, but it eases extraction of oils. Intracellular elements such as oils are difficult to extract from wet biomass with solvents without cell disruption, but are extracted more easily from freeze dried biomass (Molina et al. 1994, 2003). A balance between the drying efficiency and cost effectiveness is important for the extraction of biofuels in order to maximize the net energy output of the fuels (Li et al. 2008). The cost of drying is also an important consideration for lipid extraction from microalgae biomass powder for the food and feed industry (Li et al. 2008). Drying temperature affects the composition and total yield of lipid from the algal biomass (Widjaja et al. 2009). High concentration of TAG in the lipids could be retained at 60 °C with slight decreases in the lipid yield. Drying at above 60 °C can resulted decrease in both the concentration of TAG and lipid yield (Widjaja et al. 2009). OriginOil (a biofuel company based in Los Angeles) has also developed a wet extraction method that combines ultrasound and electromagnetic pulse induction to break the algae cell walls. In this method gaseous carbon dioxide is added to the algae solution to lower the pH that resulted in easy separation of biomass from the oil (Heger 2009).

1.4 Algal Biofuel Production Technologies

There are several pathways which are used for biofuel production by using algal bio-metabolite (bio-oils and residue). First of all, bio-metabolite is produced from chemically treated algal biomass, after that it is used for biofuel conversion.

1.4.1 Transesterification

It is an alcohol based conversion of fat or oil into esters (fatty acid methyl esters; FAMEs) and glycerol. There is nothing unique about the transesterification of algal oil compared with that of conventional vegetable oils. Alcohol such as methanol, ethanol, propanol, butanol, and amyl alcohol are used in transesterification process, especially methanol is common in use because of its low cost and its physical and chemical advantages. Three types of transesterification method (viz. acidic, alkaline, and enzymatic) can be performed for conversion of oil in its ester form. In alkali

based transesterification, sodium hydroxide, potassium hydroxide, carbonates, and some sodium and potassium alkoxides, i.e. sodium methoxide, sodium ethoxide, sodium propoxide, and sodiumbutoxide are used as alkali catalysts (Brennan and Owende 2010). The commonly used acid catalysts are sulfuric acid, sulfonic acids, and hydrochloric acid. Lipases also can be used as biocatalysts. Alkali catalyzed transesterification is much superior in use than acid catalyzed transesterification due to fast reaction. In case of high acid value of oil, acid catalyst is preferred. Some important parameters that affect the rate of transesterification reaction are: reaction temperature, type and amount of catalyst, stirring intensity, ratio of alcohol to oil, quality, and water content. After transesterification, quality of biodiesel can be improved by repeated washing with water to remove glycerol and methanol. Use of lipases offers important advantages, but its high cost is not currently feasible. Lipases can be obtained from *Rhizomucor miehei*, *Rhizopus oryzae*, *Candida antarctica*, *Candida rugosa*, *Pseudomonas cepacia*, and *Thermomyces lanuginosus*. The commercial immobilized lipase isolated from *C. antarctica* (Novozym 435) is also commonly used. Instead of using methanol, the lipase catalyzed synthesis of FAME can also be performed using alternative alcohol donors such as methyl (alkyl) acetate or dimethyl carbonate. The process of such a biodiesel synthesis is irreversible because the intermediate compound (carbonic acid monoacyl ester) immediately decomposes to carbon dioxide and an alcohol.

1.4.2 Reactive Distillation Technique

It is a modeling transesterification reaction with a reactive distillation column. Two processes such as transesterification reaction and the separation of the subsequent products take place within the same unit operation during reactive distillation. It is a more effective separation technique which aimed to maximize biodiesel yield (Mueanmas et al. 2010). This helps in reduction in capital cost which is important for sustainable development. It also offers higher conversion of oil to biodiesel with low energy consumption and solvents elimination during process.

1.4.3 Supercritical Fluid Technique

It is environment friendly, fast separation technique. Under this technique, alcoholic polarity and its dielectric constant are reduced. At this condition, alcohol can solvate triacylglyceride and form an oil/alcohol mixture which coexist in a single phase. This can yield free fatty acid alkyl esters (Saka and Kusdiana 2001).

1.4.4 Microemulsion Technique

Viscosity and other atomization problems of oil can be solved by microemulsion method (Vivekpantidar et al. 2014). It is a thermodynamically stable, transparent

colloidal dispersion. It is a mixture of oil with an ester, alcohol (butanol, hexanol, and octanol), water, and surfactant. Some problems such as incomplete combustion, nozzle failure, and carbon deposition have arisen with the use of micro-emulsified biodiesel (Koh and Ghazi 2011).

1.4.5 Biochemical Fermentation/Anaerobic Digestion

Anaerobic digestion (AD) is the conversion of algal biomass into a biogas. Biomass consists of methane (CH_4) and CO_2, with traces of hydrogen sulfide (EU 1999). It involves the breakdown of organic matter to produce a gas with an energy content of about 20–40% of the lower heating value of the feedstock. This process is applicable for wet algal biomass with high moisture content (80–90% moisture). It is not a popular pathway for algal residue conversion. Microalgae with high proportion of proteins (i.e., low C/N ratio) can affect the performance of the anaerobic digester and also result in increased ammonium production. Co-digestion with a high C/N ratio product (e.g., waste paper) can resolve this problem. High sodium ions can also trigger toxic nature to some anaerobic microorganisms, and only feasible for salt-adapted microorganisms to perform anaerobic digestion of marine algae (Brennan and Owende 2010). Some macroalgae (*Ulva lactuca*, *Gracilaria vermiculophylla*, *Saccharina latissima*) have been anaerobically digested to produce methane in a range of 0.1–0.3 LCH4/g volatile solids (VS). It has reported that microalgae (codigested with other feedstocks) such as *Spirulina platensis*, *Scenedesmus* sp., and *Chlorella* sp. yield methane in range of 0.2–0.3 L CH_4/g VS, whereas other microalgae like *Tetraselmis* sp., *Chlorella vulgaris*, *Scenedesmus obliquus*, and *Phaeodactylum tricornutum* produced methane from 0.17 to 0.28 L CH_4/g VS when digested as sole feedstock (Marzano et al. 1982; Zamalloa et al. 2012). Pretreatments (thermal, chemical, thermochemical, and biological) of algal biomass have been also studied to improve biogas production (Table 1.2).

1.4.6 Thermochemical Conversion

Thermochemical conversion covers the consumption of energy to convert organic components in biomass (a fuel source) to yield different chemical state (oil and residue). The thermochemical conversion processes involve heating of biomass at high temperatures. There are some basic approaches: such as direct combustion, gasification, thermochemical liquefaction, and pyrolysis.

1.4.6.1 Combustion

Combustion is the burning of biomass in air. It converts the chemical energy stored in the biomass into heat, mechanical power, or electricity using different process equipment. Combustion produces hot gases at temperatures around 800–1000 °C. This is an older method of utilizing biomass for obtaining energy (Goyal et al. 2008; Ryan 2009a, b).

Table 1.2 List of pretreatments of algal biomass to improve biogas yield (Magdalena et al. 2018)

Biomass	Pretreatments	Increased biogas yield
Scenedesmus sp.	Thermal (75 °C for 10 h)	58%
Scenedesmus sp.	Thermal (95 °C for 10 h)	69%
Chlorella sp.	Thermal (70 °C for 30 min)	37–48%
Stigeoclonium sp.	Thermal (130 °C for 15–30 min)	28%
Nitzschia	Thermal (130 °C for 15–30 min)	28%
Monoraphidium sp. and *Stigeoclonium* sp.	Mechanical (26.7 KJ/g TS for 30 min)	85%
Chlorella sp. and *Scenedesmus* sp.	Chemical (CaO; 4 and 10% w/w) at 25, 55 and 72 °C	25%
C. reinhardtii	Proteases (86–96% solubilization)	7%
Chlorella vulgaris	Proteases (86–96% solubilization)	51%
Scenedesmus sp.	Proteases (30% solubilization)	1.53-fold
Chlorella vulgaris and *Scenedesmus* sp.	Carbohydrases (84% and 36% solubilization)	1.2-fold

1.4.6.2 Gasification

Gasification is a thermochemical process that involves the partial oxidation of biomass into a combustible gas mixture (synthesis gas; Syngas) at high temperatures (800–1000 °C). Syngas is a mixture of CO, CO_2, H_2, CH_4, water and tar vapors (long chain aliphatics), and ash particles that contains 70–80% of the energy originally present in the biomass feedstock (Ghasemi et al. 2012). Syngas is a low calorific gas (typical 4–6 MJ m^3) that can yield fuel gases for engines and gas turbines. Gasification processes can be classified as conventional gasification and supercritical water gasification. Conventional gasification is the process to decompose dry algal biomass with low moisture content (15–20%) to the material in absence of oxygen at high temperature (800–1000 °C or higher) and pressure, which are further decomposed into small molecular combustible gas with the help of catalysts. Supercritical water gasification causes the hydrolysis of biomass components to produce smaller molecules at 347 °C with a metal catalyst or at 697 °C with a carbonaceous or alkali catalyst. Supercritical water gasification has some unique advantages over conventional gasification such as recovery of energy from wet biomass, no energy intensive drying process, high solubility of biomass components and products in supercritical water, homogeneous reaction, and simple separation of the gas products from liquid phase at the end of the reaction.

1.4.6.3 Thermochemical Liquefaction

Thermochemical liquefaction is a process that converts wet algal biomass material into liquid fuel (bio-oil) at low temperature (300–350 °C) and high pressure (5–20 MPa) using a catalyst in the presence of hydrogen (Ghasemi et al. 2012). However, liquefaction is a relatively expensive process due to the use of hydrogen. Conversion is conducted at 300 °C, accommodating high moisture content biomass. With the help of a catalyst, the process utilizes the high water activity in sub-critical

conditions to decompose biomass materials down to smaller molecule materials with a higher energy density.

1.4.6.4 Pyrolysis (Thermal Cracking)

Pyrolysis is a thermochemical process to convert dry biomass to bio-oil, syngas, and charcoal by heating the biomass at medium to high temperatures (350–700 °C) in absence of oxygen with the aid of a catalyst (Goyal et al. 2008). It involves cleavage of chemical bonds to yield small molecules. Flash pyrolysis at moderate temperature (500–600 °C), short hot vapor residence time (about 1 s) is a viable technique for future replacement of fossil fuels with biomass derived bio-oil. The biomass-to-liquid conversion ratio (95.5%) can be achieved through pyrolysis (Demirbas 2006). Carbon monoxide, alkanes, alkenes, charcoal, phenol formaldehyde resins, carboxylic acid, and wastewater are common byproducts of pyrolysis. Metallic salts are used as a catalyst in many studies. A few studies on pyrolysis method for the production of microalgae derived fuel oil have been studied (Miao and Wu 2004; Miao et al. 2004). High quality bio-oil was obtained through fast pyrolysis of *C. protothecoides* (Yield of 18%) and *Microcystis aeruginosa* (Yield of 24%) at temperature of 500 °C (Miao et al. 2004). The quality and quantity of the pyrolysis products depend on various factors, such as reaction temperature, heating rate, pressure, reaction time, etc. Compared to other conversion technologies, pyrolysis of algal biomass is quite extensive and has achieved promising and reliable outcomes that could lead to commercial exploitation.

1.5 Use of Natural Resources

To achieve maximum biomass growth regarding both technical and economic performance towards biofuel production, resource factors must be appropriately matched. Algal biofuels productions require resource factors such as suitable climate, land, CO_2 supply, water management, and other nutrients for sustainable use and cost effectiveness. The sustainability of algal production systems can be evaluated using a system of social, environmental, and economic indicators.

1.5.1 Climate

Climate and temperature elements determine the overall algal productivity. Solar insolation, temperature, precipitation, evaporation, and weather events are climatic factors to affect viability of algal biomass production. The availability of adequate sunlight, climate suitability, and temperature are key factors to determine economic feasibility of algal growth. Photoautotrophic microalgae growth in open and closed cultivation systems depends on the availability of abundant sunlight. The average seasonal insolation factor is the rate-limiting factor for autotrophic algal productivity. The daily, seasonal, and annual variation in solar insolation on spatial surface area of cultivation systems is needed to achieve a set amount of product, downstream

processing design capacity, the amount of CO_2, and the amount of culture. In addition to insolation other climate-related factors (temperature and cloud cover, precipitation, wind, etc.) will also affect both the productivity and reliability of algal production. The optimized temperature for algal biomass growth is in between 20 and 35 °C, though strains vary in temperature tolerance. Higher temperatures can lead to reduced productivity rates, while very low temperature can cause slower algal growth (Pate 2013). As a result, lower latitude areas are more preferable for algal growth due to more stable temperature range (Pate et al. 2011; Lundquist et al. 2010; Pate 2013; Quinn et al. 2012). According to requirement the operating temperature range can be adjusted through the use of different open, closed, and hybrid cultivation systems for algal culture. In open pond systems, optimum temperature can be affected by evaporative water loss, solar gain during the day, pond depth, pond mixing, radioactive heat loss at night, the thermal coupling, and bidirectional heat flow through pond. Comparatively closed photobioreactors (PBRs) are less sensitive to climate change than open ponds. However, temperature monitoring is needed in PBRs due to limited evaporative cooling. Availability of temperature and sunlight (seasonally and annually) will directly affect the algal productivity, whereas other climate changes (precipitation, evaporation, and severe weather) will affect quality and demand of water in open systems.

1.5.2 Water

Suitable water is an important factor for algal cultivation. It is dependent on geographical and local conditions. Most of the areas of the country are suited for algal culture due to the highest solar resource but it is subject to more limited water supplies (Wigmosta et al. 2011; Venteris et al. 2013). Instead of geographic location, algae-based biofuels will also depend on water use and consumption in the cultivation systems (photoautotrophic/heterotrophic/mixotrophic) and growth system (open vs. closed systems). Different degrees of water usage are incurred if there is a need to replace water lost by evaporation in open pond systems, or to use water for evaporative cooling in closed systems. Mixotrophic and heterotrophic systems must also require water used for upstream production of organic carbon feedstock. Cultivation of algal feedstock can be dependent on less, the same, or more water than terrestrial biofuel crops. It all depends on cultivation process, co-products, and location of systems (Batan et al. 2013).

1.5.3 Land

Open and closed systems will require land availability for photosynthesis-based biomass feedstock. Various factors such as physical, social, economic, environmental, legal, and political factors affect land availability for algal production. It has demonstrated that open pond system is required approx. 5.5% land area of the Continental United States (CONUS) to generate 220×10^9 L/year of oil through

open pond production (Wigmosta et al. (2011) and photobioreactor is required 1.853 $\times$ 10^8 acres area in the CONUS plus Hawaii to produce 315 billion gallon/year (BGY) of algal lipid production (Quinn et al. 2012). Topography and soil limit the land availability for algae farming in open pond systems. Soils porosity and permeability affect the construction costs and design of open systems. Topography is the limiting factor for open pond systems because the installation of large shallow ponds requires relatively flat terrain. The bulk density of soil (quality indicator) below the liners could influence the future productivity of the soil for the environmental sustainability of algal biofuels. Biofuel production at coastal sites could also be influenced by land prices and availability. Land desirable for development and for publicly beneficial reasons may not be seen as suitable for biofuel production.

1.5.4 Nutrients

Availability and cost of nutrients (i.e., nitrogen, phosphorus, and potassium) play an important role in commercial viability for algae growth (Venteris et al. 2014; Pate 2013; Williams and Laurens 2010). Pate (2013) has been estimated that 7 million metric tons of nitrogen and 1.03 metric tons of phosphorus will be required for algal biofuel production in range from 4.5 BGY to 12 BGY. The requirement of nutrient determined by hydrothermal liquefaction with combined catalytic hydrothermal gasification (CHG) has resulted in improvement on nitrogen (34%) and decrease in phosphorus consumption (52%) as compared to another method of lipid extraction (Venteris et al. 2014).

1.5.5 Carbon Dioxide

Atmospheric CO_2 is a limiting factor for efficient algal production due to slow diffusion rates. Autotrophic microalgae cultivation scalability and operating expense are influenced by CO_2 availability and cost of delivery. CO_2 waste gas could be used a potential source for low-cost algal cultivation. Algae can use emitted CO_2 to convert into organic molecules that can be used as biofuel, building blocks for the biotechnology industry, and energy. Thus algae production could be efficiently used to reduce fossil carbon emission. Algae production does not directly involve in fossil carbon sequestration, rather provides carbon capture and reuse in the form of fuels derived from the algal biomass. Mitigation of CO_2 emissions from large industrial plants, including coal and natural gas combustion plants are already operated under the research area of carbon capture and utilization as a climate change abatement strategy (Rubin et al. 2005; Campbell et al. 2008).

Several studies have been focused on the availability of carbon dioxide from stationary sources for algal cultivation (Pate et al. 2011; Quinn et al. 2012; Venteris et al. 2014). Estimated 10 billion gallons of fuel could be produced using sufficient CO_2 from stationary sources (Pate et al. 2011). This amount of fuel production account to approx. 20% of the U.S. waste carbon emitted per year (Venteris et al.

2014). A major challenge for algae is capturing a major percentage of CO_2 emissions from any given source. It is estimated that 2200 acres of algae cultivation area is required to utilize the fully CO_2 emitted from the flue gas of a natural-gas-fired power plant. Algae can only utilize total generated CO_2 during the photosynthetically active sunlight hours (Brune et al. 2009). Due to CO_2 off-gassing during non-sunlight hours and the unavoidable losses of CO_2 during algae production, CO_2 emissions offset will be limited to an estimated 20–30% of the total power plant emissions. Recent work of Davis et al. (2016) suggests that major percentage utilization of total emissions is possible through conducting primary CO_2 capture with an absorber. The system could capture 80% from low-concentration sources (flue gas with 4–4.5% CO_2 concentration) and 90% from higher-concentration sources (coal). Thus the overall capture and use of the CO_2 is assumed as 70–80%.

1.6 Potential Environmental Effect During Production of Algal Biofuel

Cultivation of microalgae as biological resource for the production of biofuel has been a broad scope of research. Large-scale production of algal biofuel using different resources has been shown to have both positive and negative impacts on the environment (Hannon et al. 2010a, b). Limited studies that have been done so far discussed the scale up of cultivation system and also environmental impacts that could occur. This chapter includes the possible environmental impacts from algal biofuel production. Potential effect of anthropogenic activity on land, water, and air quality and also emission of greenhouse gas (GHG), biodiversity, effect of genetically engineered algae organisms, waste products, diseases, pathogens, and toxins.

1.6.1 Change in Anthropogenic Activity on Land

Change in anthropogenic activities is mainly change in land-use. It is very important for sustainable development of algal biofuel because of associated environmental changes like emission of GHG, alterations in ecosystem and biodiversity, deforestation, and urbanization. The effect of algal biofuel production will depend partially on the type of land-use, how much the land got disturbed, and also for how long the change persist. Large-scale production of algal biofuels will need substantial land area to develop facilities. Land for algal cultivation does not require fertile soil so it leads to cost-effective biofuel production, agricultural land, industrial land and land for residential use are not required. Moreover, forest land conversion is also not suitable because of the high costs of clearing. Algal biofuels production may involve brownfields, scrubland, abandoned farmland; also, unproductive farmland and coastal islands could also be used. Physical, an algal biofuel producing company, utilizing fallow land in Hawaii, which was previously used for pineapple plantation

(Weblink n.d.). Competing land demands could also influence land for algal biofuels production.

Land-use changes could affect the net GHG emissions of biofuels and also carbon sequestration and it is depending on the land conversion type and prior land-use (NRC 2004). For algae cultivation, converting pastureland to algal ponds enhances GHG emissions. On the other hand, algal cultivation in ponds on spoiled land that is not storing much carbon and emits less GHG. Algae producing protein and lipid potentially could replace soybean or other terrestrial crops (Wijffels and Barbosa 2010). Cultivating algal biofuels will require different work frame than regular crop farming. Above discussion illustrates a potential land-use change by conversion of cropland to algal ponds for biofuel production.

1.6.2 Water Quality

Algal biofuel production may recover or damage water quality depending on the input resource and management. Large-scale production of algal biofuels required sufficient water for culture and also concern regarding eutrophication of water, groundwater contamination, and salinity of water which released to natural environments. Algae can also be used to remove nutrients from municipal waste and other liquid wastes if cultivated with proper management. It may provide potential benefits to water quality as compared to runoff of herbicides and insecticides compared to corn-grain ethanol or soybean-based biodiesel production. Water requirement for algae cultivation can be reclaimed and reused to reduce and effluent from production plant also could be recycled and use to produce of biogas (Davis et al. 2011). Effluent containing nitrogen and phosphorous concentration depending on the nutrients utilize by cultivated algal biomass (Sturm and Lamer 2011). Eutrophication occurs when a water body receives high concentrations of inorganic nutrients which stimulates algal growth and results in huge algal biomass production. High nutrient load could lead to anoxia in the deep portion of lakes or in hypoxia in the receiving water bodies and lead to affect ecological community (Scheffer et al. 1997; Reynolds et al. 2002).

1.6.3 Air Quality and Greenhouse Gas Emission

One of the main motives to develop biofuel production strategies is to mitigate GHG. In this regard life-cycle assessment (LCA) of GHG emissions of algal biofuel production is critically reviewed. Sander and Murthy (2010) utilized algae biomass for corn in ethanol plants. Replacing feedstock corn with oil-extracted algae for ethanol results in a negative carbon balance. However, it is hard to conclude that algae based on recycling CO_2 and producing biogas have net negative GHG emissions. N_2O could be emitted from cultivation systems and emissions would need to be quantified in the future. Fagerstone et al. (2011) shows quantification of N_2O emissions from algal culture under laboratory conditions. In this study of

Nannochloropsis salina with nitrate as a nitrogen source, elevated N_2O emissions were observed under a nitrogen headspace (photobioreactor simulation) during dark periods, but N_2O emissions were low during light periods. Proper management of the algae biofuel production systems, which would prevent senescence of algae and maintain aerobic conditions in ponds, likely would keep N_2O emissions to low levels.

1.6.4 Aquatic and Terrestrial Biodiversity

Microalgae are cosmopolitan in their biogeographical distributions and therefore they have their broad habitat range. However, they are not necessarily found in every location so their distribution is often mosaic-like (Hoffmann 1996). Coastal marine macroalgae inclined to less distributed than microalgae and phytoplanktonic forms. Macroalgae have restricted range of temperature, light, substratum, and nutrient preferences. Whitford (1983) explains that species of freshwater algae may appear to be infrequent for several reasons and this suggests that few rare species of algae could be displaced by invasive algae used to produce biofuel feedstocks. For biofuel production from algae, open culture system in natural environment such as open ponds system is very common. Release may occur during the feedstock production stage or possibly during the harvesting or drying stages. Characteristics like rapid growth, vegetative propagation, pest resistance can make algal species desirable for the production of biofuel. In open pond system, several exotic species could disturb the coherence of local and regional ecosystems (Ryan 2009a, b).

The outline of landscape conversion for any new setup could affect terrestrial species diversity through three distinct mechanisms that not only apply for production of algal biofuel but also for other energy production (McCabe 1994; DOE 2009; Garvin et al. 2011).

(1) Movement of terrestrial vegetation and wildlife habitat from the facility area and replacement with a pond or photobioreactor containing a monoculture or a few species of algae. (2) Reduction in local wildlife habitat area below the threshold which will be needed for the species. (3) Fragmentation of wildlife habitat and migration corridors should be disrupted. The magnitude of land requirements and the types of conversions affect the magnitude of probable effects on ecological populations and communities. There are some studies that focus primarily on terrestrial ecosystems and terrestrial plant biofuel feedstocks, rather than on aquatic systems and algal feedstocks (Fargione et al. 2009; Fletcher et al. 2011; Wiens et al. 2011).

1.6.5 Effect of Genetically Modified Organisms

The sustainability goals for genetically engineered algae likely would include two other issues: (1) to minimize dissemination of genetically engineered algae (2) an establishing method to determine whether an observed effect was caused by a

genetically engineered alga. Abundance of genetically engineered algae released to water could be measured through species-specific tests if the species were not native. Moreover, some modified traits, such as altered antennae might be detected microscopically and thus would be quantifiable in water. Particular DNA sequences might also be measureable. Moreover, markers could be tagged to algae to allow easy measurement in specific media.

1.6.6 Waste Products

Sustainability of algal biofuel manufacturing process is enhanced by recycling of raw materials and minimization of waste. Solid waste from algal biofuel production processes is generated as sludge from which the volatile organic acids have been converted to CO_2 and methane. The produced methane can be useful as a fuel supplement. Golueke et al. (1957) reported average NH_4 concentrations of digested sludge in the range of 1600–1850 mg/L for anaerobic digestion of algae (Sukias and Tanner 2005; Sukias and Craggs 2011).

Nutrients recycling is obvious mitigation for waste generation. Maximizing recycling would also reduce the need for waste disposal. In this regard an algal biofuel production company, Algenol, is making efforts to use recycled seawater waste for cultivation of algae. Information is needed about the types and rate of waste generation for most algal biofuel production processes. When and if processes move toward commercialization, state and local regulations will govern the acceptable disposal of waste, which will necessarily be well characterized by then.

1.6.7 Diseases, Pathogens, and Toxins

As algal biofuel production technology rapidly increases, many environmental anxieties are put forwarded, if concerns are not addressed they may be raised as to the potential social and health related risks. There are still challenges to overcome the technology from pilot scale to industrial scale production of biofuel from algae. However the cost effectiveness of biofuel industry needs to be considered. Some of the environmental risks might further require exploratory assessment and subsequent monitoring to ensure that they do not become sustainability concerns if algal biofuel production is scaled up.

1.7 Applications, Process Constraints, and Future Needs

A sustainability evaluation of biodiesel production from microalgae is very important. Although microalgae appear to be superior in some respects to other currently used feedstocks, the development of large-scale microalgae production systems still needs further research. An outline for selecting sustainability, three points must be

taken into account—sustainability of this process, economic concern, and environmental concern.

1.7.1 Sustainability of Algae for Biofuel

Under the liquid transportation category, algal biofuel can provide viable third generation feedstock. However, for commercial industry development, sustainability with advanced technological and economic issues appropriate to algal based fuel must be taken care of. To meet this vantage and challenges, attentions must be paid on three combined approaches. First, for sequential biomass processing and also to obtain numerous high-value products, an integrated algal biorefinery approach should be followed which would ultimately result in the financial sustainability to the algal biofuel production. Second, an integrated renewable energy park (IREP) approach is proposed for combining various renewable energy industries established in diverse locations. This will help for synergistic and effective electricity and liquid biofuel production with net carbon emissions will be zero. This could avoid numerous sustainability issues such as productive usage of agricultural land and fossil fuel usage. The algal biofuel industry can be linked with the other pharmaceutical and neutraceutical industries for the sustainable development.

1.7.2 Socioeconomic Considerations

To support design of sustainable energy systems, socioeconomic indicators can be used. These indicators representative of energy security, external trade, profitability, resource conservation, and social acceptability have not yet been well checked in sustainability assessments for commercial algal biodiesel producing facilities. Socioeconomic indicators that have been showed commercially and measured at the laboratory level. Moreover, to describe socioeconomic sustainability of algal biofuel production, it is important to describe factors affecting this process and also other indicators that could be measured during production. Indicators which are described in literature are mainly profitability indicators, net present value (NPV), return on investment (ROI), fossil energy return on investment (EROI), and the resource conservation indicator. These indicators have apparent sustainability targets and very perfect to design sustainable algal biofuel systems. Factors affecting these indicators are set-up, type of process, and financial expectations. Food-related co-products from algae could enhance food security. The energy security indicators and fuel price instability and exterior trade indicators terms of trade and trade volume cannot be anticipated into the future with precision prior to commercialization.

Furthermore, advancement to achieve sustainability of algal biofuel would only be possible when both environmental sustainability indicators as well as socioeconomic sustainability indicators should contribute together.

1.7.3 Environmental Considerations

There are three dimensions of sustainability and they basically are very much interrelated. They are economy, environment, and society. Sustainability occurs at the intersection of the three domains. A system becomes more sustainable when all three domains, as represented by the intersection of the three domains and also easy to evaluate relative sustainability of a selected system. Microalgal biomass can be pyrolyzed to produce different kinds of other biofuels such as hydrocarbons, biogas, and biohydrogen. Microalgae cultivation opens new possibilities for its combination with processes, such as wastewater and flue gas treatments, removal and sequestration of carbon dioxide, with the potential for reducing additional environmental impacts. Various researchers demonstrated use of microalgae for pollution control and production of useful products and energy cogeneration combustion (Hodaifa et al. 2008; Murakami et al. 1998). Thus microalgae can be environmentally sustainable and cost effective for the production of biofuels. Microalgae are also an important part of a biotechnology supply chain that can yield many of the basic chemicals necessary for the development. The growth and cultivation of microalgae needs less land area as compared to other agricultural feedstocks. Mata et al. (2010) reported, microalgae cultivation utilize 49 percent or 132 times less when compared to rapeseed and soybean crops, respectively. Also, factors like quantity of nutrients available, solar irradiation, and other environmental conditions have great role to select the most adequate species for biofuels production (Mata et al. 2010). Other algal production facilities may use nonnative species or species that have been selected and bred or genetically modified for desirable characteristics for algal biofuel production.

1.7.4 Future Opportunities for Algal Biofuel to Improve Sustainability

Algal biofuel production process includes growth and higher biomass production, separation, and conversion of microalgae biomass. At the end of separating process the preferred biofuel product and significant portion of byproduct remains. For the environmental sustainability of the process it is important that byproducts have a useful and safe purpose. Menetrez (2010) evaluated environmental impact on the cellulosic ethanol production from algae carbohydrate with the help of genetically modified organisms. The waste outlet contains biological toxins, allergens, carcinogens produced by these microorganisms. Bioprospecting is important to identify algal species that have desired traits like high lipid content, growth rates, growth densities, and/or the presence of valuable co-products while growing on low-cost media. Despite the potential of these strategies, it is difficult to identify species. To overcome this hurdle genetic engineering and breeding will be required to bring these strains to economic viability.

The genetically modified algal strain have improved lipid biogenesis and enhance crop protection along with valuable enzyme and protein as co-products. Moreover,

risks to human health through exposure to GMO lead to toxigenicity and allergenic responses. GMO also impacts environment mainly for future of biodiversity via unintended transfer of transgenes. Among sustainable energy source, algae and the feedstocks have great potential to replace petroleum-based fuels. Currently, algae industry focuses not only on production of high volumes of biofuel at relatively low cost but also byproducts which may be used as pharmaceuticals and nutritional supplements. Environmental impact of algae renewable fuel production on different resources includes the treatment of wastewater, carbon dioxide capture from power plants, cosmetics, pharmaceuticals, organic fertilizers, soil nutrient recovery, and aquaculture. Ultimately, to satisfy the high demand of alternative energy source and also to reduce fossil fuel dependency makes this technology imperious. Commercialization of algal based biofuels will have a reflective future impact on society. Waste products produced by this process that are currently discharged into the environment will be utilized to produce much needed renewable energy sources. No sustainable technology is without its trials but blind promotion of those technologies without honest consideration of the long-term implications may lead to the acceptance of strategies whose long-term consequences outweigh their short-term benefits. Even given these uncertainties, we believe that fuel production from algae can be cost competitive and widely scalable and deployable in the next coming years, but only if we continue to expand our understanding of these amazing organisms as we expand our ability to engineer them for the specific task of developing a new energy industry. Now it is time to initiate the development of an algae industry and evaluation strategies to make algae-based fuels costs competitive with petroleum.

1.8 Conclusion

Algae are recognized as one of the oldest life-forms and also as the worldwide fastest growth plants. These phototrophic organisms require for optimal growth sunlight, CO_2 from the air, water, inorganic salts (N, P, K), and temperature in the range of 20–30 °C. Microalgae can fix CO_2 from three different sources: atmosphere, discharge gases from heavy industry and from soluble carbonates. Microalgal biomass contains approximately 50% of carbon by dry weight. Producing 100 ton of algal biomass fixes roughly 183 ton of CO_2. Depending onspecies, microalgae produce many different kinds of lipids, hydrocarbons, and other complex oils. In general, many algae species have the oil content ranging from 20 to 50% by dry weight of biomass. The lipid and fatty acid contents of microalgae vary in accordance with culture conditions. It is possible to increase the lipid concentration by optimizing the growth determining factors almost up to 80%. This chapter examines three aspects of microalgae production that will strongly influence the future sustainability of algal biofuel production: the energy and carbon balance, environmental impacts, and production costs. Against each of these aspects microalgae production presents a mixed picture. A positive energy balance will require technological advances and highly optimized production systems. The mitigation of environmental impacts, and

in particular water management, presents both challenges and opportunities, many of which can only be resolved at the local level. Existing cost estimates need to be improved and this will require empirical data on the performance of systems designed specifically to produce biofuels. At the current time it appears that the sustainable production of biofuels from microalgae requires a leap of faith, but there are nonetheless grounds for optimism. The diversity of algae species is such that it is highly likely that new applications and products will be found. As experience with algal cultivation increases it may also be found that biofuels have a role to play. An important caveat to all these conclusions is that they reflect the state of the existing academic literature, and this is inevitably an imperfect reflection of the status of the sector. It is quite possible that many of the challenges identified are being addressed, but that the information about how this is being achieved is yet to make it into the public domain.

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Microbial and Biotechnological Advancement in Biogas Production

2

Muhammad Naveed Anwar, Muneeba Shabbir, Hira Saif, Simal Hassan Khan, Eza Tahir, Ajwa Tahir, Zaib Naeem, Mohammad Rehan, and Abdul-Sattar Nizami

Abstract

Energy crisis, solid waste management, ever-increasing CO_2 and methane levels, unemployment, deforestation, increased energy generation cost, and depleting fossil fuels are some current challenges faced by developing countries. The biogas production is a sustainable, lenient, and affordable approach to address these issues. This chapter focuses on the history of biogas digesters and their evolution, feasible techniques for biogas production, and methods to enhance biogas quality. It highlights the advantages and limitations of fixed dome digester, floating drum digester, and plug flow digester. Organic waste such as animal dung, food waste, agricultural waste, municipal solid waste, industrial waste, and sewage sludge can be used as feedstock to produce biogas in digesters. Acetic acid produced from glucose and water in acetogenesis process is transformed into methane and by-products through methanogenesis. The efficient production of biogas is carried out by a complex microbial process in which an appropriate environment is necessary for the multiplication of microbes and their proper functioning. Biogas generated at low temperatures using psychrophilic enzymes has a low methane content; however, other factors such as pH, oxygen content, and salt concentration also affect microbial activities and hence the quality of the biogas. The electrical energy produced by biogas from agricultural waste feed-stock is carbon zero. In Asia, biogas production is the need of the time and will not only contribute towards a low carbon economy but also will address the longstanding issue of deforestation and environmental pollution. If increasing energy demands of a growing population in Asia and Africa are addressed

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through this renewable approach, then it will enhance the energy security and environment integrity of these two continents.

2.1 Introduction

The increasing solid waste generation and its mismanagement is a global issue, causing serious environmental pollution and human health issues (Zavodska 2003). Uncontrolled urban sprawl has further added salt to injury and made solid waste management (SWM) a challenging problem (Ghose et al. 2006). Legal, financial, and economic aspects associated with it have highlighted this issue in developing countries on a regional, local, and national level (Palabiyik 2002). There cannot be a single panacea to this problem. Every city and state has to develop its SWM system according to the physical and geological setup coupled with the nature of waste being produced (Monavari et al. 2016). Developed countries have successfully developed an integrated waste collection and disposal system, while developing countries, mainly due to lack of capacity building and technological advancements, are left with the only option of open dumping. SWM system of Pakistan, like other developing countries' systems, is also not immune to these mismanagement issues and serves as grave environmental hazards of the time. Even the most advanced urban localities have 57% of their housing societies equipped with a formal disposal system. The situation is more alarming in small towns where only 2/5th of the population has access to a decent waste collection system (Khan et al. 2016).

This open dumping or somewhat decent disposal techniques not only pose severe environmental hazards in the form of land, water, and air pollution but also additionally release around 590–880 million tons of methane. This is the outcome of the biological decomposition and degradation of organic matter in waste through microbial activities (GATE, GTZ 2007). The methane contributes 17% of total greenhouse gas emissions, making it a potent greenhouse gas. The global warming potential of methane is 21% over CO_2. Since 1750, its concentration has increased from 15% to 1800 ppm. In terms of total radiative forcing (TDF), it accounts for 20% (IPCC 2001, 2007). China, India, United States, European Union, and Brazil are ranked as the top economies with maximum methane emissions (Climate Analysis Indicator Tool 6.0 Version 2009). Agriculture is the primary source of GHG emissions, and it contributes 14% of the total methane emission. Livestock and paddy rice are also leading causes of methane emission, and Livestock makes up 37% of anthropogenic methane emissions (Korres et al. 2013).

Figure 2.1 provides an account of various GHGs emitters in Pakistan. The energy sector has been responsible for most of the GHG emissions in the past, present, and the trend seems to remain valid as far as future predictions are concerned. The agriculture sector is the second-largest contributor to GHG emissions. Emissions from waste and energy production can be significantly reduced through waste to

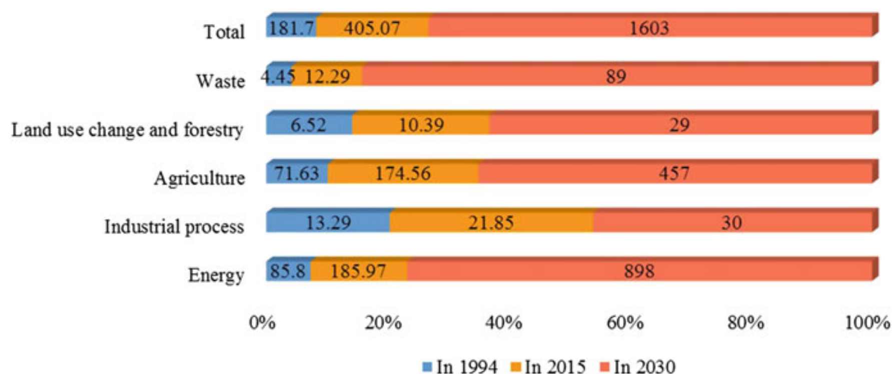


Fig. 2.1 Sector-wise projection of GHG emissions in Pakistan, Mt CO_2-equivalent (source: MPDR-Ministry of Planning Development and Reform 2010)

energy options employing biogas plants. The situation is more promising since Pakistan has all the necessary raw materials for energy production through waste decomposition.

Traditional ways of burning dung cakes, firewood, straw, and agriculture residue with inadequate ventilation systems produce toxic particles and affect human health severely. Indoor air pollution from biomass burning and poor ventilation in homes had increased risk of respiratory infections among children, chronic pulmonary diseases in adults, and other diseases including less birth weight, tuberculosis, ear infection, and cataracts (Bajgain and Shakya 2005). More specifically, 1–2 million deaths were caused because of the burning of solid fuels in Sub-Saharan Africa and have contributed 3–4% of global mortality in 2000 (REN 21 2005).

Developing countries, including Pakistan, are suffering from an energy crisis and ever-increasing gap between energy demand and supply. In order to meet the energy deficit, Pakistan spends around US$7 billion annually on fossil fuels import amounting to 40% of total imports (Heedge and Pandey 2008). The imported fossil fuel is then burnt in various thermal power plants to produce energy. Predictions have shown that energy demands will rise three-folds in 2050; however, the energy supplies do not seem to be increasing proportionately (Asif 2009).

Hence, to meet the ever-growing energy demands and alleviation of the economic situation of Pakistan, and most of the developing countries, the diversified energy mix having alternative energy resources is imperative. In 2010–2011, an economic survey was conducted in Pakistan, and it revealed that biomass is the most readily available and cheapest renewable resource. There is plenty of agri-waste in all rural areas of Pakistan. 652 million kg of animal manure is produced daily by cattle and buffaloes with the potential to generate around 16.3 million m^3 of biogas daily and 21 million tons of bio-fertilizer per year. These biogas energy generation plants can contribute significantly to overcome the energy needs of Pakistan and other developing countries since they require low initial capital cost and can be started with a

low budget even on a community level. Biogas plants can serve as good alternative energy for 70% of the population living in rural areas (Korres et al. 2013).

Most pressing issues across the globe such as solid waste management, energy demands, and supply gap, rising methane and CO_2 levels, dependence on fossil fuels, global warming, and life-threatening diseases can be tackled through a single panacea: Biogas production. For instance, if one city is divided into ten blocks or segments and the SWM system of each block is designed individually, then it will allow the concerned departments to manage solid waste effectively. It will not only minimize the consumption of fuel during transportation of solid waste from one block to the last corner of the city, also resulting in traffic congestion and air pollution, but will also provide the concerned block with the valuable resources. Such as, the block can install large-scale biogas plants and hence can become self-sustainable in terms of energy resources. Additionally, it will help improve the solid waste management and reduce the air emissions in general and Carbon emissions emitted during the waste collection and transportation. These kinds of block scaled waste to energy plants when installed on a massive scale, at the national level, can play an instrumental role in addressing global warming, economic crisis, and other environmental crisis of grave concern such as air pollution and solid waste management globally.

Biogas comprises methane 50–70%, carbon dioxide 30–50%, and minor amounts of nitrogen 0–3%, water vapors 5–10%, oxygen 0–1%, hydrogen sulfide 0–10,000 ppm, ammonia, hydrocarbons, and siloxanes. However, the biogas efficiency can be further increased by filtering unwanted gases (Angelidaki et al. 2018). This chapter emphasizes on microbial and biotechnological advancements in biogas production, an evolution of different feedstock choices and operational setups over time. Fixed dome digesters are the earliest form of digesters that were commonly used in developing countries. Floating drum digester is the modified form of fixed dome digester which became operational in 1962. Another form of digester plug flow digester is a cheap digester with a shorter lifespan. Biogas production is initiated and carried out by complex microbial process and a lot of operating conditions affect microbial activity and efficiency such as temperature, oxygen content, pH, substrate, and salt concentration. Different active microbes are required at different stages of biogas production. Biogas is attaining the attention of the public and government because of its feasibility, cost-effectiveness, and market opportunities as an alternate source of renewable energy. European Union is quickly switching to biogas to fulfill growing energy demands sustainably. Malaysia is also promoting small renewable energy programs to gain energy specifically from biomass and municipal waste. Hence, biogas can be, and is, an important means of earning the carbon credits through a reduction in GHGs emissions from conventional solid waste management systems and yielding clean renewable energy instead of employing the fossil fuels' combustion for energy production.

2.2 Biogas

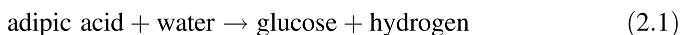
Anaerobic digestion of organic materials produces biogas which is mainly composed of methane and carbon dioxide, with other trace gases such as hydrogen sulfide (McKendry 2002; Hiremath et al. 2009). The process takes place in the absence of oxygen and bacteria degrade organic materials (Incinerators 1977). Biogas composition varies with operating conditions of the digester and nature of the feedstock. The major portion of biogas is composed of 50–75% methane (v/v) and 25–50% carbon dioxide (v/v). Other trace components (v/v) such as water vapor (1–5%), nitrogen (0–5%), ammonia (0–500 ppm) and hydrogen sulfide (0–5000 ppm) are also present (Braun 2007).

2.2.1 Biogas Production Process

Biogas production is a multi-stage and complex process that involves a consortium of bacteria (Fig. 2.2). The followings are the steps involved in the production of biogas.

2.2.1.1 Hydrolysis

In the first step, the complex components of waste or organic matter (carbohydrates, lipids, and proteins) are degraded into simpler compounds (sugars, amino acids, alcohols, and fatty acids) by cellulolytic, lipolytic, and proteolytic bacteria, respectively. Both facultative and obligatory anaerobes such as *Bifidobacterium*, *Megasphaera*, *Sporobacterium*, *Sphingomonas*, *Propionibacterium*, *Lactobacillus* are commonly used in hydrolysis (Khanal 2011).



$$\text{adipic acid} + \text{water} \rightarrow \text{glucose} + \text{hydrogen} \tag{2.1}$$

(Korres et al. 2013)

2.2.1.2 Acidogenesis

Acidogenesis or acid production is the second stage in biogas production. The products of the hydrolysis stage are further broken down in the second step by acidogenic bacteria producing volatile fatty acids (short-chain organic acids), e.g. propionic acid, lactic acid, and butyric acid. Meanwhile, carbon dioxide and methane are also produced. Like hydrolysis, both facultative and obligatory anaerobes such as *Escherichia coli*, *Staphylococcus* spp., *Lactobacillus* spp., *Corynebacterium* spp., *Desulfovibrio* spp., *Bifidobacterium* spp., *Peptococcus anaerobius*, *Clostridium* spp. take part in this phase (Metcalf 2003; Khanal 2011).

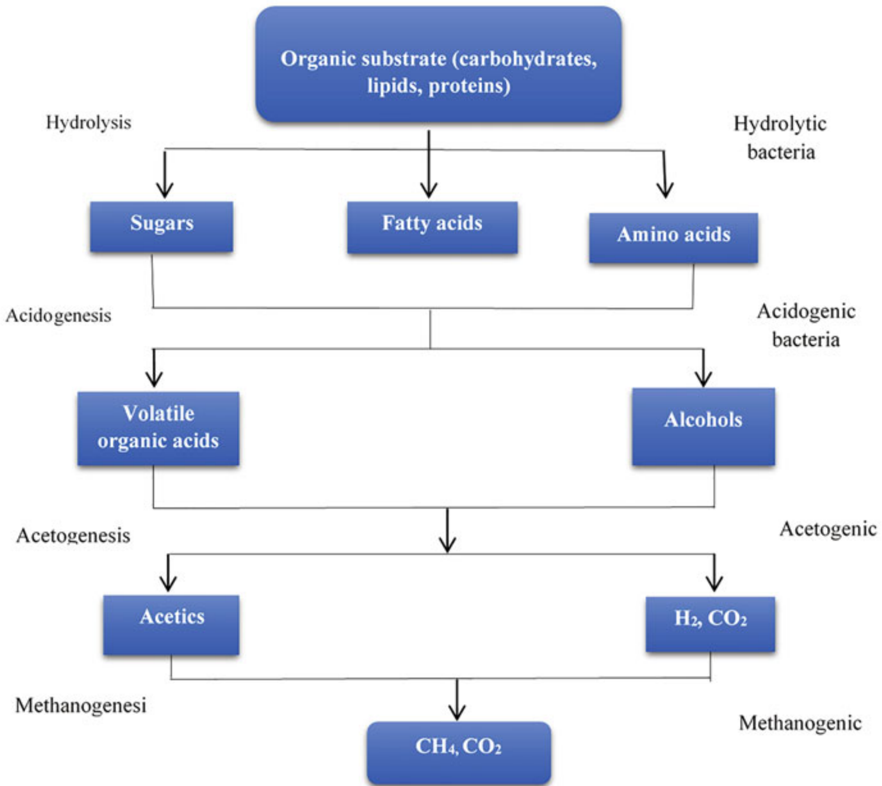


Fig. 2.2 Anaerobic digestion process. Modified from Mao et al. (2015)

$$\text{glucose} \leftrightarrow \text{ethanol} + \text{carbon dioxide} \tag{2.2}$$

$$\text{glucose} + \text{hydrogen} \leftrightarrow \text{propionic acid} + \text{water} \tag{2.3}$$

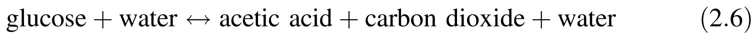
$$\text{glucose} \rightarrow \text{acetic acid} \tag{2.4}$$

(Korres et al. 2013)

2.2.1.3 Acetogenesis

The third step of biogas production is acetogenesis or acetic acid production. At this phase, acetogenic bacteria breakdown volatile fatty acids and ethanol, produced in acedogenesis (second step), into acetate/acetic acid (CH_3COO^-), carbon dioxide, and hydrogen. Acetic acid, hydrogen, and carbon dioxide are produced by acetogenic bacteria, e.g. *Syntrobacterwolinii* and *Syntrophomonaswolfei* (Vavilin et al. 2008).

$$\text{pripionate} + \text{water} \leftrightarrow \text{acetate} + \text{hydrgen ion} + \text{hydrogen carbonate} + \text{hydrogen} \tag{2.5}$$



$$\text{glucose} + \text{water} \leftrightarrow \text{acetic acid} + \text{carbon dioxide} + \text{water} \tag{2.6}$$

$$\text{glucose} + \text{water} \leftrightarrow \text{acetic acid} + \text{carbon dioxide} + \text{hydrogen} \tag{2.7}$$

(Korres et al. 2013)

2.2.1.4 Methanogenesis

The fourth and last stage involves the transformation of CO_2, H_2, and acetate into a blend of CH_4 and CO_2 through acetotrophic and hydrogenotrophic methanogens. Acetotrophic methanogenesis is the process in which acetotrophic methanogens use acetate as a substrate. Hydrogenotrophic methanogenesis is the utilization of the H_2 as an electron donor to reduce the CO_2 by hydrogenotrophic methanogens. Bacteria from the genera *Methanosaeta*, *Methanosarcina*, *Methanobacterium formicidcum*, and *Methanobacterium ruminantium* are involved at this stage (Alexander 1978).

$$\text{carbon dioxide} + \text{hydrogen} \rightarrow \text{methane} + \text{water} \tag{2.8}$$

$$\text{acetic acid} \rightarrow \text{methane} + \text{carbon dioxide} \tag{2.9}$$

$$\text{ethanol} + \text{carbon dioxide} \rightarrow \text{methane} + \text{acetic acid} \tag{2.10}$$

(Korres et al. 2013)

2.2.2 History

Before the birth of Christ, renewable resources, e.g. wastewater was used for the energy supply, e.g. combustible gas was used to heat water by the Persians and Assyrians (He 2010). The Sumerians around 3000 BC practiced anaerobic cleansing of waste. Pliny, the Roman scholar, explained the flickering lights which appeared under the surface of swamps (Deublein and Steinhauser 2011). In 1804, the chemical composition of inflammable air was identified to be CH_4 by Dalton. The first scientist to produce biogas was Gayon (Tietjen 1975). More scientific and systematic research to understand and comprehend the process of anaerobic fermentation kicked start in the nineteenth century (Deublein and Steinhauser 2011). However, in the nineteenth century, the risk of failure was higher and anaerobic digesters were smaller in capacity. The technology of anaerobic digestion moved from laboratory experiments to field applications. In France, the simple air-tight chamber was used to treat sewage. In England, the septic tank was used to treat wastewater (McCarty 2001; Gijzen 2002). In Exeter, street lamps were run on gas produced from waste-water (Fig. 2.3). Germany first sold methane to the public in 1923. Using biogas was very popular until the Second World War. During the period 1930–1940, biogas was produced using agricultural waste. The importance of biogas was reduced around

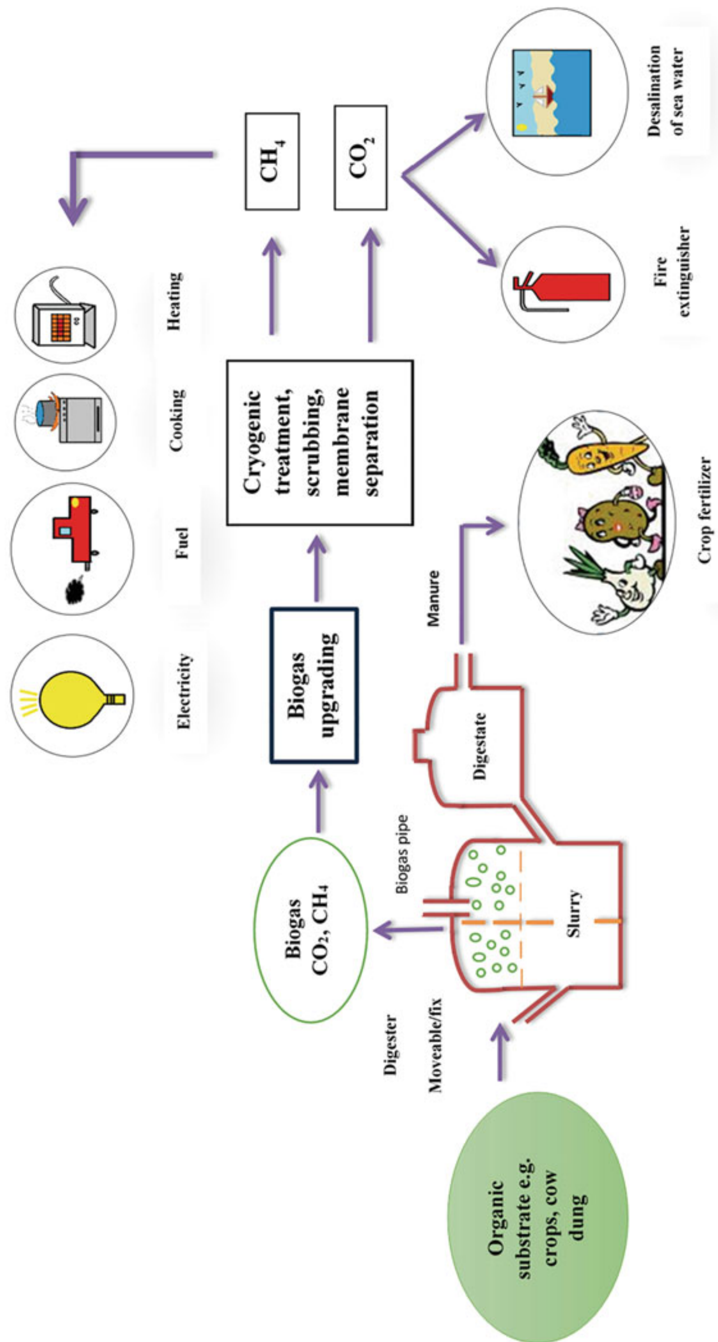


Fig. 2.3 Schematic diagram of biogas production and utilization

1955 due to the excessive availability of oil. In the 1990s, profitability and prevention of waste production, pollution prevention, were reasons for the stimulation of biogas technology (Deublein and Steinhauser 2011). In the twentieth century, Germans invented "Imhoff digesters" for sewage sludge treatment. Using the same technology, larger anaerobic digesters were used for the treatment of sewage sludge for public national gas grid in 1920 (Bond and Templeton 2011).

2.2.3 Types of Digesters

2.2.3.1 Fixed Dome Digesters

Fixed dome digesters are being operated in China since the early twentieth century and commonly used in developing countries: Ghana, Kenya, and India (Akinbami et al. 2001; Chen et al. 2008; Bond and Templeton 2011; Nzila et al. 2012). They are also called "Hydraulic" or "Chinese" digesters (Santerre and Smith 1982). Clay, concrete, and cement are used for the inner walls of the digester to make it impermeable (Chen et al. 2008). An inlet pipe is used to fill the digester chamber and biogas is collected in the storage section. Due to the height difference between the expansion chamber and slurry in the digester, gas pressure is produced. Some substrate is introduced into the expansion chamber as the produced gas requires space and presses it. A digester is immediately filled again with slurry as the gas releases (Sasse et al. 1991). They are built underground (Santerre and Smith 1982) and thus, require skilled labor incurring higher costs (Veen et al. 2009). Another cost factor is the transportation of construction materials in case biogas plants are constructed in remote areas (Pérez et al. 2014). The main advantage of this model lies in its low maintenance costs as it is immovable, and the absence of metallic parts makes it immune to rusting. This model does not produce gas at constant volume and the pressure of the gas is not maintained, thus less inefficient to run a gas generator, and gas heater. The construction material is prone to crack, which leads to digester failures. The lifespan is over three years for these types of digesters (Cheng et al. 2014).

2.2.3.2 Floating Drum Digesters

This digester was built in 1962 and its original name was Khadi and Village Industries Commission (KVIC). This model is widely used in India (Rajendran et al. 2012). Like fixed dome reactors, these digesters are also constructed underground using steel and concrete. The digester is cylindrical and there is a moveable inverted drum for the collection of gas, which is made of PVC or steel. The drum moves upward as the gas is produced and falls back as the gas is drawn off. (Singh and Sooch 2004; Rajendran et al. 2012). There are no mechanisms for heating or mixing in these digesters (Cheng et al. 2014; Surendra et al. 2014). This model has an advantage of the production of the gas at constant volumes, as the drum maintains it. Moreover, stored gas volume can be determined easily, and the overall digester's operations are simple. However, the downsides affiliated with this type of digester are its high construction costs due to expensive raw materials (steel). Since steel is

prone to corrosion, so it needs regular maintenance and monitoring. All these factors contribute to a lifespan of 8 years. Furthermore, presence of any fibrous material hinders the movement of the drum (Rajendran et al. 2012). Because of inefficient agitation, deposition of solids occurs restraining the exposure of the substrate to microorganisms (Singh et al. 1997).

2.2.3.3 Plug Flow Digesters

In 1957, South Africa used this type of digester design for the first time (Ghosh and Bhattacherjee 2013). High-density polyethylene is used for construction (Lansing et al. 2008). This model consists of a narrow and long tank. The inlet and outlet are located opposite to each other. The parts above ground are inlet and outlet, while other parts are kept in an inclined position underground. The two processes, acidogenesis and methanogenesis are separated due to the inclined position creating a two-phase system. The digestate flows at the other end as a new substrate is added through the inlet. A shed roof or gable is used to cover the digester to avoid temperature fluctuations and provide insulation (An et al. 1997; Bouallagui et al. 2003; Ferrer et al. 2009, 2011). The comparatively lesser lifespan makes it economically less viable. The shorter lifespan is because of the fragile nature of PVC which is subjected to extreme conditions and forceful mechanical contact (Nzila et al. 2012). This model has the advantage of simple design, adaptability to extreme conditions, easy handling, easy installation, reasonable retention time, and low capital cost (Ghosh and Bhattacherjee 2013). There are no moving parts so failure risks are reduced (Singh et al. 1997). However, certain disadvantages such as less biogas production, the slow solid conversion, and absence of agitation are also associated with this model (Ghosh and Bhattacherjee 2013).

2.3 Range of Waste Utilization in Biogas Production

A diverse range of waste types can be utilized for waste to energy technologies like anaerobic digestion (AD). Different types of organic waste include food waste, animal dung or livestock manure, agricultural waste, sewage sludge, industrial waste, and the organic portion of municipal solid waste (Khalil et al. 2019). These waste types can be very harmful to the environment and human beings when openly dumped (Raheem et al. 2016). Table 2.1 compares the biogas production from different types of waste, digester and energy potential (Fig. 2.4).

The biogas generation potential of cattle dung varies from 56 to 68 m^3, fats biogas potential varies from 826 to 1200 m^3, and that of pig slurry varies from 11 to 25 m^3. The biogas yield and electricity generation of every waste is given per ton of fresh matter (Achinas et al. 2017).

Table 2.1 Comparison of different types of digesters (source: Korres et al. 2013)

Classification basis	Substrate feeding		Substrate DS content		AD process technology	
Types	Batch digester	Continuous digester	Dry digester	Wet digester	Single stage	Two stage
Advantages	No need for pumping, mixing, stirring	Capital cost is low, design and operation are simple	Simpler pre-treatment, retention of biomass is higher	Freshwater dilutes inhibitors	Less technical failure, simple design	Recirculation increases degradation Less susceptible to failure
Disadvantages	Biogas yield is low, clogging and channeling	Production of larger VFAs and acidification	Expensive, complex, mixing and material handling is difficult	Crop digestion leads to scum formation, risk of short-circuiting	Retention time is high, formation of scum and foam	Construction and maintenance are expensive, solid particles need to be removed

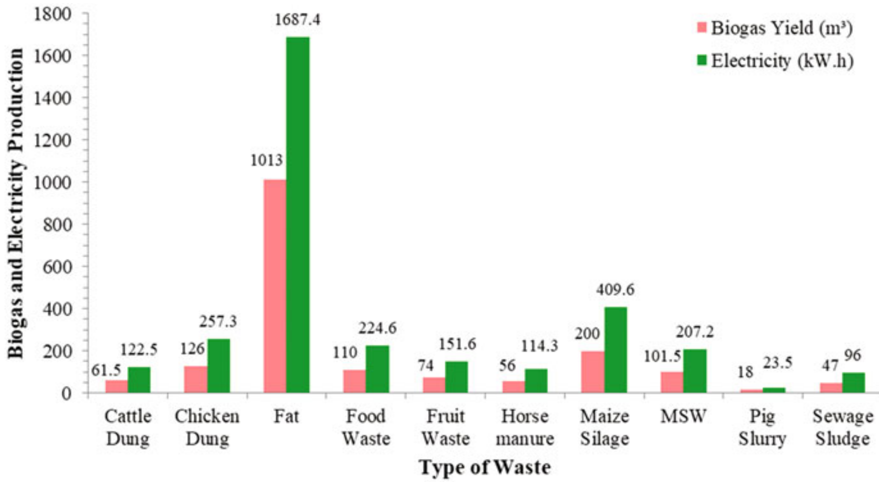


Fig. 2.4 Biogas yield and electricity production potential of different feedstocks (source: Achinas et al. 2017)

2.3.1 Animal Dung

Animal dung holds a significant amount of nitrogen and phosphorus. The high concentration of these elements disturbs the nutrient cycle and degrades the environment. It comprises some other harmful residues like heavy metals, hormones, antibiotics, and microbes, which not only pollute the environment but also become the sources of hazardous human diseases. This is one of the leading causes of air, water, and soil contamination. Therefore, the conversion of livestock waste to useable energy form, i.e. biogas is the most environmentally friendly and sustainable means of its utilization (Abdeshahian et al. 2016). In Asian countries, the most common sources of animal dung are poultry, cattle, sheep, goats, horses, camels, and buffaloes, and their number is also growing day by day. The amount of manure, an animal excretes, is directly linked to its age, weight, and feeding behavior. Total waste can be estimated by multiplying waste excreted (by an animal in a year) with a total number of animals. The poultry sector is the chief sector in all the Asian countries like India, Pakistan, Bangladesh, Nepal, and Sri Lanka with 656, 530, 242, 24, and 15 million birds, respectively, produced per annum. The dung produced per animal in the poultry sector is 0.05 tons annually. On the other hand, manure produced by a buffalo and a cow is 6.1 and 6.0 tons per year, respectively. Their headcount is 30 million buffaloes and 31.8 million cattle in Pakistan, whereas 98.6 million buffaloes and 174 million cattle in India. Therefore, the poultry birds may be high but waste excretion is most elevated in the case of buffaloes and cows that are noteworthy in biogas generation (Raheem et al. 2016). Poultry waste contains dry organic matter, feathers, head, feet, offal, and blood that are carbohydrates, proteins, and lipids. Manure is composed of 35% dry material, 24% protein, and 18% fats. As

poultry waste is produced in a large amount, about 97.5×10^6 cubic meter of biogas can be produced by utilizing all poultry waste in an AD process (Arshad et al. 2018).

2.3.2 Agriculture Residues

Agricultural crop residues are one of the essential sources of biofuel energy. It contains woody substances, crops remain, and fruits and vegetable residues. Crop residues can be gathered from two sites: first in the agricultural field where crops are grown and harvested, and second from the factories or industries where crops are processed. The residues collected from the field include leaves, twigs, seeds, and straw, whereas those obtained from processing sites include bagasse, roots, and husk, etc. Crops like wheat, rice, corn, sugarcane, and cotton produce large quantities of residues beneficial for biogas generation. Other than croplands, processing factories produce fruits and vegetable wastes and their derivatives, which are equally important. Asia accounts for 61% of the vegetable production worldwide, i.e. largest source. About 30% of the global fruit supply comes from India, Brazil, and China. Industries, from all over the world, produce large quantities of these wastes including 81 million tonnes of potatoes, 37 million tonnes of tomatoes, 29 million tonnes of citrus, 25 million tonnes of bananas, 17 million tonnes of apples and grapes (Paudel et al. 2017). Grassland is also getting attention because of its beneficiary use in biogas production. Grass silage has a high number of volatile solids which is directly proportional to biogas yield. About 300 m^3 of methane can be generated by using one ton of volatile solid (Nizami et al. 2009).

Typically, agricultural straw has a low nitrogen content and they are lignocellulosic, which make them difficult to digest in the process of biogas generation. Their complex structure is the main limitation of ineffective processing. To prevent this problem, several researches have suggested pre-treatment methods (Onthong and Juntarachat 2017). De-lignification treatment is a suggested method to break down the complicated polymer structures of agricultural and forest wastes. It can be carried out through several chemicals, mechanical, biological, thermal, and combined approaches as well (Yu et al. 2019).

2.3.3 Municipal Solid Waste

Municipal solid waste (MSW) is continuously increasing with a rapidly growing population globally. It is estimated that the annual production of MSW will grow to nearly 27 billion tons per year until the year 2050 (Ali et al. 2019). The organic portion of MSW is approximately 46%, comprising food waste, kitchen waste, and garden waste. Other than an organic fraction, paper waste makes up about 17%, plastic waste 10%, glass and metals 9%, and others 18%. One ton of organic portion of MSW can produce around 200 m^3 of biogas which can generate 400 kWh of electricity (Tyagi et al. 2018). Food waste is a major portion of this organic waste. It has a high carbon-nitrogen ratio as well as volatile solids and total solids ratio.

According to USEPA, 1000 kg of food waste have the potential of producing 376 m^3 of biogas (Ali et al. 2019). The highest food waste generation rate is 0.5 kg per capita per day in Canada, then 0.37 and 0.3 kg per capita per day in England and the United States, respectively. In developing countries, India, China, and Brazil have the maximum food waste generation rates (Dung et al. 2014).

2.3.4 Co-digestion of Multiple Wastes

The efficiency of anaerobic digesters can be increased by using more than one waste type in a single digester, also known as co-digestion. It is reported that up to 43% methane production can be improved because of the synergetic effects (Horváth et al. 2016). In Germany, grass and maize silage are commonly digested together in biogas plants (Nizami et al. 2009). Co-digestion of poultry waste and rice husk is also suggested to produce energy and considered being cost beneficent and environmentally sustainable procedure (Arshad et al. 2018). About 220 kg of rice husk can be produced from one ton of rice paddy, and its potential of producing energy can be estimated from the fact that one ton of rice husk can generate up to 570 kWh electricity (Ali et al. 2016). Agricultural residues combined with livestock waste and food waste is also a very promising co-digestion strategy to enhance environmentally friendly biogas generation (Hagos et al. 2017).

2.4 Microbial and Biological Advancement

A complex microbial process is responsible for efficient biogas generation through the action of numerous microbial species utilizing different substrates as feedstock. These organisms need to work collectively to attain maximum output. Thus, a simple principle is to introduce anaerobic microorganisms for the degradation of the organic material which is kept in an air-tight container for ensuring that no oxygen enters it (Schnürer and Jarvis 2018).

2.4.1 Environmental Influences

These microorganisms require an appropriate environment for multiplication and accomplishment of their function. Some significant ecological aspects of development are:

- Temperature
- O_2 concentration
- pH
- Salt content

Varieties of microbes require varied suitable environmental factors central to their optimum growth. It briefly describes some of these factors below:

2.4.1.1 Temperature

The maximum temperature variation depends on the adapted temperature range of the microorganism. Biogas production involves diverse microorganisms, and they have variable optimum temperatures, that is why their responses also vary to some extent. The biogas process typically runs at a temperature range of about 30–40 °C or 50–60 °C. Biogas generation can also take place at psychrophilic temperatures but might lead to a reduced CH_4 production rate depending on the type of process (Bohn et al. 2007; McKeown et al. 2009; Dhaked et al. 2010). At elevated temperatures, there are examples of methanogens that can survive at up to 110 °C (Chaban et al. 2006), whereas stable biogas production mechanisms do not appear to function above 60–70 °C (Scherer et al. 2000). In case of a temperature higher than 60 °C, the activity of methanogens is greatly decreased compared to acid producers, which usually leads to the fatty acids' buildup in the biogas process (Scherer et al. 2000). Thermophilic organisms endure high temperatures (up to 60 °C approx.) although their maximum growth occurs at the mesophilic temperatures.

2.4.1.2 Oxygen Concentration

The importance of oxygen concentration is variable for the diverse microbial communities involved in the production of biogas. Microorganisms are generally divided into several groups in relation to their suitable oxygen content requirement (Fig. 2.5).

2.4.1.3 pH

Different microorganisms involved in the generation of biogas correspond significantly differently to a range of suitable pH for their optimum multiplication and development (Table 2.2).

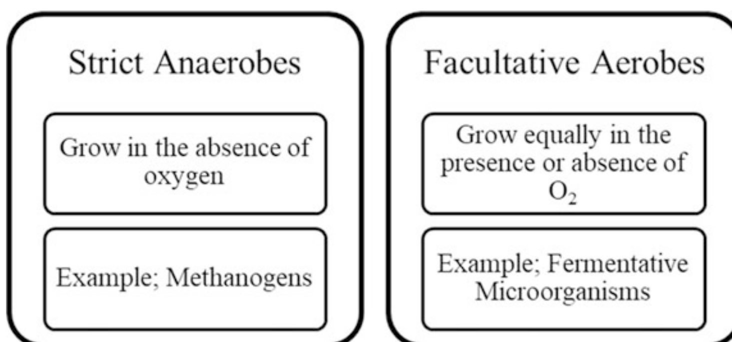


Fig. 2.5 Classification of microbes involved in biogas process based on oxygen content (Ağdağ and Sponza 2004)

Table 2.2 Suitable pH values for microorganisms involved in the biogas process

pH requirement	Microorganisms	pH value
Low pH/acidic conditions	Fermentative and acid-forming microorganisms, acidophilic methanogens	5 or less than 5
High pH/alkaline conditions	Alkaliphilic methanogens	10 or greater than 10
Neutral pH	Methanogens	7

Although the growth of most methanogens occurs at pH of around 7, neutral, these may also survive at other pH environments as well (Whitman et al. 2006). Some well-acknowledged microbial strains such as acid-loving methanogens grow at pH less than 5—around 4.7 (Bräuer et al. 2006) and alkali-loving methanogens whose growth occurs around pH 10 (Mathrani et al. 1988). In Sweden, many biogas production methods are operational at pH around 8. In addition, research writings likewise encompass illustrations of biogas production approaches functioning at less than 6 pH (Savant et al. 2002).

2.4.1.4 Salts

All microorganisms need salts for proper functioning. Salts comprise essential building blocks, i.e. Na, K, and Cl; for the microbes. These substances are naturally available in most substrates, and their separate addition in the biogas process is not needed. Salts usually have an antibacterial behavior and act as inhibitors for the growth of bacteria. Methanogens are greatly influenced by the high salt content involved in the biogas generation (Chen et al. 2008).

2.4.2 Substrates

Carbon-based waste is utilized to produce biogas and accounts for several substrates for different microbes. The greater the types of components in the waste used, the higher the availability to a variety of microbes for biogas production is. Substrate composition is significant both for the output gas produced and the qualitative aspects of it.

2.4.2.1 Substrate-Specific Enzymes and Microbial Strains

Enzymes are usually substrate-specific, and the microbial strains that provide these enzymes are also particular (Fig. 2.6). Biogas process involves several types of substrates as raw materials for the generation of biogas, using several microorganisms that are responsible for providing enzymes specific to substrates. The flow diagrams below illustrate the different kinds of raw materials utilized as substrates by several enzymes that act upon them for attaining the desired biogas products. These different substrate types have a different chemical composition and require specific microorganisms that offer enzymes needed for their degradation and formation of new products.

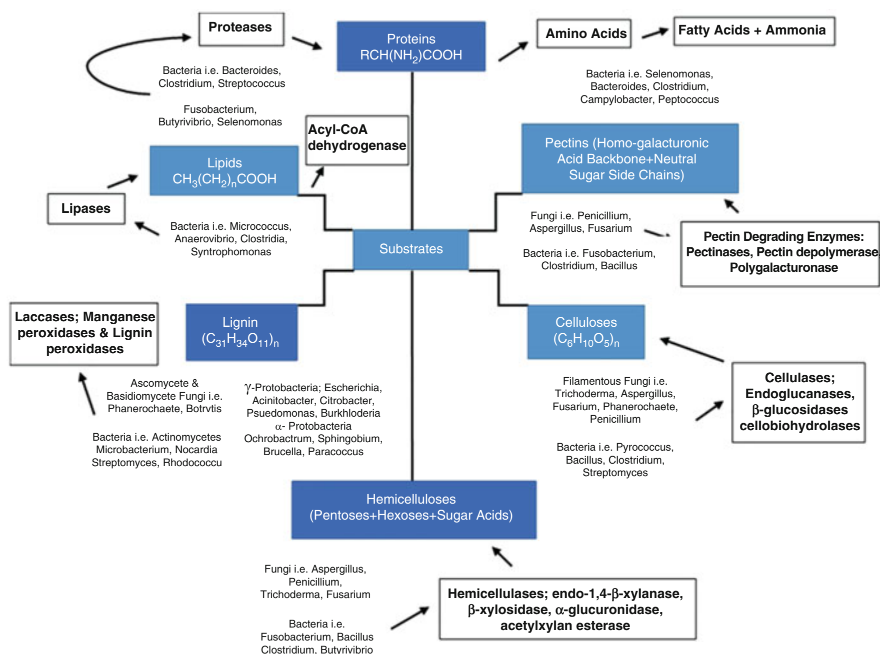


Fig. 2.6 Different substrates, their chemical composition and Microbial strains that produce specific enzymes for the substrate degradation

As described in the diagram above, every substrate gets converted into a unique bio-product by the action of enzyme groups released by various specific microbial strains. The table given below gives an insight into a series of different substrate groups along with their chemical composition, including the amount of carbon and nitrogen contents. Several various sources of these substrates are also shown, i.e. various industries (pulp, paper, and food), wastage, and some other materials. Substrates require optimum temperatures for their degradation because of their enzyme and microbe specific nature; that is why the temperature requirements of each of the substrate groups listed in the Table 2.3 are clearly mentioned.

2.4.3 Active Microorganisms at Different Stages of Biogas Process

The existence of numerous diverse microbiological species is vital for the functioning of the biogas generation process and for producing end products: methane/ hydrogen gas. All the microbes involved in action need to work similarly collectively (Angelidaki et al. 2011; Schnürer et al. 2016).

The microbial strains that are dynamically involved in the entire process are illustrated as Fig. 2.7.

Table 2.3 Different substrates used in the production of biogas along with their composition, sources, and temperature requirements

Substrates employed for biogas production		Composition	Sources	Temperature requirement	References
Food waste and waste from food industries		Proteins, fats, carbohydrates, and various trace elements	Food industries and restaurants	Mesophilic and thermophilic	Gunaseelan (1997), Capson-Tojo et al. (2016), Zhang et al. (2016)
Manure	*Solid Manure*	Highly carbon enriched with greater dry solids amount up to 70%. Straw + hay + feces	Cattle yields, pigs, horse, and poultry	Mesophilic	Nasir et al. (2012), Hadin and Eriksson (2016)
	Liquid Manure	Greater nitrogen content along with a lesser number of dry solids up to 10%.			
Crops and crop residues		Dry solid amount approx. 10% to 50%. Cellulose + hemicellulose + lignin (e.g. straw)	Potatoes, corn, silage, sugar beets, grass, grain, fruit, straw, foliage	Self-heating of the process, i.e. Planned process temperature of 35–39 °C, increased to 42–49 °C with temperature increase of 0.15–0.5 °C/day	Braun et al. (2008), Lindorfer et al. (2008)
Waste from slaughterhouses and fisheries		Fats and proteins/high C/N ratio	Blood, guts/colon or soft tissue, fish wastes, wastewater	Mesophilic and thermophilic	Salminen and Rintala (2002), Franke-Whittle and Insam (2013), Bustillo-Lecompte and Mehrvar (2015), Hamawand (2015)
Sewage sludge		Populations of aerobic microorganisms with cell walls of complex proteins and carbohydrates	Sewage waste	Thermophilic	Anjum et al. (2016), Demirbas et al. (2016)
Algae	*Macro-algae*	Dry solids content about 10–15%, low lignin content, 20–70% fat depending upon growth conditions	Food, fat-accumulating species of algae and EPS	Co-digestion under mesophilic + thermophilic settings	Chen et al. (2015), Zhang et al. (2016)
	Micro-algae				

Wastewater and Sludge from Pulp and Paper Industries	Some soluble organic material and various potentially inhibiting organic substances, i.e. bleaches (peroxides), chlorinated compounds, sulfur compounds (sulfate, sulfite, etc.) tannins, terpenes, and LCFA	Pulp and paper industry	Mesophilic	Meyer and Edwards (2014), Kamali et al. (2016), Zhang et al. (2016)
Stillage	Rich in protein, also releases furfurals, small phenolic compounds	Ethanol production, untreated waste, wheat, and other crops or cellulose-rich residues	Mesophilic	Torry-Smith et al. (2003), Jönsson and Martín (2016)
Wood products	High carbon content and releases phenolic compounds	Untreated spruce and pine, Salix and poplar	Thermophilic	Ericsson et al. (2014)

Sources: Van Soest et al. (1991), Saha (2000), Brummell (2006), and Kumar et al. (2008)

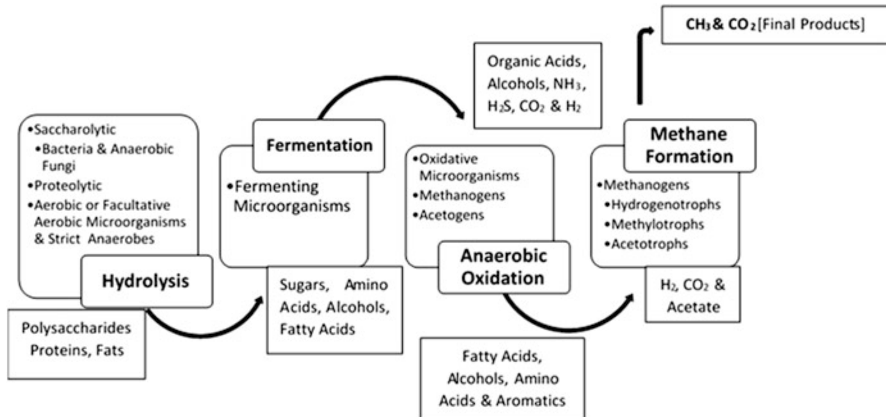


Fig. 2.7 Active microorganisms at different stages of the biogas process described stepwise (Schnürer and Jarvis 2018)

The flow diagram above is an illustration of the whole biogas process, with each step distinguished from the other. The kinds of microbes involved in each stage are shown along with the substrate compounds that, after degradation, change their form and get converted into smaller molecular constituents. The microbial strains involved are also sensitive to the type of molecule or compound of the given substrate material. In the biogas production, the action of microbial groups is limited to a specific stage of the process.

2.4.3.1 The Methanogenesis Pathways

Methanogens are classified depending on the type of substrates utilized by them, i.e. either hydrogenotrophs, methylotrophs, or acetotrophs. Similarly, different biochemical pathways are used by each type to produce methane (Korres et al. 2013), depicted as Fig. 2.8.

The two pathways shown above depict that the products formed depend on the type of microorganism. The acetotrophic methanogens directly convert into the desired product biogas, and carbon dioxide is produced as a byproduct. Whereas in the second pathway shown above, the two microbial types represented first convert acetate to hydrogen gas and carbon dioxide, and afterward, it is obtained as biogas in the second step.

2.4.4 Evolution of Microorganisms

The great diversity of microbial species offers the opportunity to discover innovative metabolic functions in recently discovered strains and to explore for enzymes of specific, identified function via screening for genes coding of such enzymes (Krause et al. 2006; Simon and Daniel 2009; Uchiyama and Miyazaki 2009; Dugat-Bony et al. 2012). Two common domains in the biogas microbial communities are bacteria

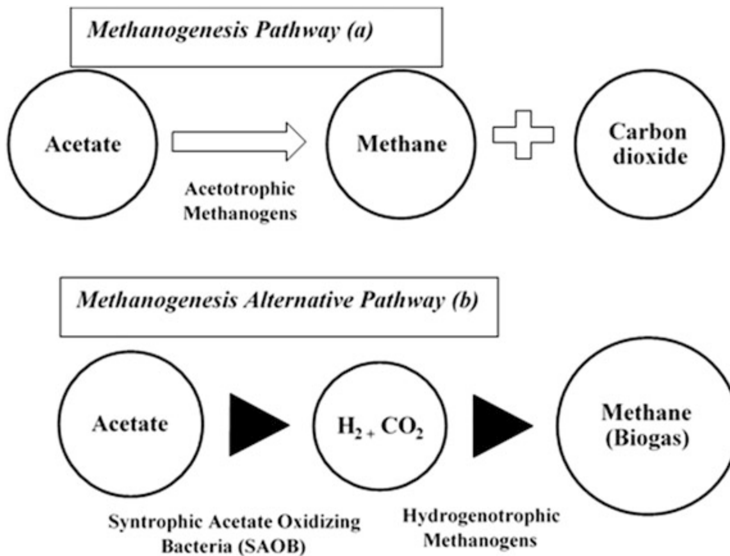


Fig. 2.8 Different biochemical pathways for methane production

and archaea counting methanogens. Eukaryotes, i.e. anaerobic fungi, along with viruses, have also been discovered. Representatives of the bacterial phyla, i.e. *Firmicutes* and *Bacteroidetes* are dominant in the production of biogas. However, members of the phyla *Chloroflexi* and *Proteobacteria* are likewise identified in the process, though less abundant (Schnürer et al. 2016). Methanogens found in the biogas process belong to phylum *Euryarchaeota*, whereas fungi identified belong to *Neocallimastigomycota*. Some microorganisms existing are still not being identified as of today.

Usually, microorganisms that can be typically seen in the AD process comprise *Clostridium* spp., *Actinomyces*, *Escherichia coli*, *Micrococcus* spp., *Peptococcus anaerobius*, *Desulfovibrio* spp., *Lactobacillus*, *Bifidobacterium* spp., *Staphylococcus* spp., *Corynebacterium* spp., *Bacillus* spp., *Methanospirillum, Methanobacterium*, *Methanosarcina*, *Methanobacillus*, *Methanothrix Methanococcus*, and *Methanosaeta* (Grangeiro et al. 2019).

2.4.5 Biofuel Generations

There are a few biofuel generations that have been developed over time based on the substrate material being utilized. The first generation of biofuel was formed by employing starch, edible oil, and sugars; then comes the second generation of biofuels that being contrarily generated by utilizing non-edible biomass; next, third biofuel generation is formed by using algae; whereas fourth biofuel generational group is formed by the capturing of CO_2 or by employing certain other innovative

technological advances. However, presently produced biofuel contains a significant proportion of first generational groups (Bhatia et al. 2017).

2.4.6 Microbial Advancement in Production of Biogas (Biohydrogen)

Various microorganisms have been stated as beneficial to produce biohydrogen. Commercial production of biohydrogen for economic wellbeing requires the consumption of lignocellulose. Jiang along with his coworkers utilized acid hydrolyzed sugarcane bagasse as a raw material for the fermentation of *Clostridium butyricum* and reported 2.06 mol H_2/mole-total sugar. To overcome the problem of temperature variances between saccharification and fermentation, in a complex process consuming lignocellulose, a thermophilic strain was isolated by researchers, i.e. *Thermoanaerobacterium thermosaccharolyticum* for carrying out fermentation at higher temperatures and reported 6.38 mmol H_2/g. *Clostridium thermocellum* is also capable of performing biohydrogen fermentation at high temperature but its productivity is low. Then, Wang and his coworkers employed a coculture of *Clostridium acetobutylicum X9* in addition to *Ethanoigenens harbinense B49* for hydrogen generation consuming cellulose as a carbon source, and 8.1 mmole H_2/g was reported. For enhancing the efficiency of biohydrogen production, Cha with his coworkers engineered *Caldicellulosiruptor bescii* by removing lactate dehydrogenase, and the resultant strain was capable of producing 21–34% more hydrogen (Bhatia et al. 2017).

2.4.7 Enhancing the Efficiency of Biogas Process

Some ways of improving the yield of biogas process are stated as follows:

2.4.7.1 Bio-augmentation

Lately, various efforts have been made for improving the biogas production by direct addition of microbes or enzymes, with some success (Schnürer et al. 2016). This bio-augmentation has been brought into light chiefly for improvement in the hydrolytic step and enhancement in the degradation of lignocellulose. For instance, the degradation productivity of a substrate, i.e. wheat straw was augmented with the addition of cellulose-degrading bacterium *Clostridium cellulolyticum*.

2.4.7.2 Addition of Microorganisms

Naturally, present microorganisms or artificially prepared ones could also be cultured collectively, for the advancement of some actions, i.e. rise in the consumption range of substrate, enhance the yield, and enable the reclamation procedure (Bhatia et al. 2017). Improvement in the stable and efficient production of CH_4 at elevated NH_3 contents is also attempted, and for this purpose adding the methanogen. *Methanoculleus bourgensis* resulted in increased methane production

and a stable process. A greater CH_4 output was also achieved by subsequently adding H_2 producer organism, i.e. acetogenic *Enterobacter cloacae*, whereas a quicker degradation of fat was attained after adding *Clostridium lundense*.

2.5 Opportunities

The development and expansion of non-fossilized clean energy source are of significance to the energy security and environmental integrity (Bong et al. 2017). The considerable development in the manufacturing of goods is relieved by the worldwide population increase and advancement in technology, hence leading to an exponential rise in the economy of the industrialized nations. It should also be considered that all the raw materials and fuels, which are extracted from the earth, convert into emissions or waste at some point. The aim should be to keep the volume of these emissions as low as possible, which ultimately would reduce the negative impact on the environment (Barik 2018). There is an increasing concern over the high production rate of organic waste due to rapid urbanization and population growth around the globe. Biogas is of great interest among the renewable energy available due to its ability to treat organic waste and generate power addressing both concerns at the same time.

Production of biogas using the animal manure produced in farms represents an additional energy source for generating heat and electricity (Ramos-Suárez et al. 2019). Thus, it is not wrong to say that biogas is a plethora of opportunities in the case of both, meeting demands contrast to the energy crisis and a solution to the immense waste disposal problem.

2.5.1 Factual Productivity Through Biogas

Biogas production, utilization, and renewable energy, cost-effectiveness, business and commercial potential, and market principles are emphasized as a standard criterion for renewable energy technologies in economic arguments and various policy documents. Biogas occurs as a major part of it (Table 2.4).

Considering a net amount of waste to be 2.12 billion metric tons per year (as stated by the reports of World Bank 2018) we surely have a huge amount of waste to be generated every year (Levine 2018). This amount is contributed as 0.74 (kg) of footprint per person per day. Almost 70% of the net amount of waste is organic in kilograms nature, which makes up to 1.484 billion metric tons of waste (Gautam et al. 2019). Out of the 70% organic waste (The World Bank), the expected biogas production mainly depends on the contents of both dry matter (DM) and lignin (% of DM) of the organic waste (research gate). So, rounding off, we can conclude that almost 60% of organic waste can be utilized for biogas production. Ending up, we have nearly 1.3 billion tons of waste feasible to produce biogas. Considering 100% of organic waste produces 153 m^3 ton-1 (Al-Addous et al. 2019), 60% of organic waste will end up in 91.8 m^3 ton-1 production of biogas. 1.3 billion tons of 60% organic waste would be ending up in 119.71 billion m^3 of biogas. The

Table 2.4 Energy consumption

Constituent	Usage in 2016 (in million tons of oil equivalent)	%Annual increase/decrease from 2005 to 2016
Total amount of energy	13,276	1.8
Natural gas	3204	2.3
Oil	4418	1.1
Nuclear electricity	592	−0.5
Coal	3732	1.6
Hydroelectricity	910	3.0
Wind and solar electricity	292	25.3
Biomass, geothermal, and other renewable electricity	127	7.4

Source: Gautam et al. (2019)

LCA to be carried where we have 1 m^3 of biogas corresponds to 6.29 barrels of oil. The cost of one barrel of oil is equal to 53.76 USD. So, the 119.71 billion m^3 of biogas produced is equivalent to 753 billion barrels of oil and the cost is 39,909 billion USD approximately. This gives us a scenario about the financial benefit of production of such amount of biogas.

2.5.1.1 European Union and Europe

Biogas production has seen an unremarkable growth in last years around Europe (Table 2.5). It was mainly driven by favorable support schemes in various European Union Member States.

The rate of production of biogas has improved in the European Union. An increase in biogas production is also associated with renewable energy policies, environmental, social, and financial benefits, and it reached 18 billion m^3 methane (654 PJ) in 2015, which represents half of the global biogas production (Scarlat et al. 2018).

2.5.1.2 Malaysia

Malaysia has been continuously inferring Renewable Energy-promoting policies and actions, for instance, the Small Renewable Energy Program, National Green Policy 2009, National Renewable Energy Act 011, Feed-in Tariff (FiT) mechanisms, Renewable Energy business fund, and Green Technology Financial Schemes.

Precisely, the Renewable Energy resources included energy from biomass, municipal waste, and biogas. Biogas is relatively essential in a waste management perspective as it can offer a win–win scenario towards the nation's efforts to achieve energy security along with combating waste accumulation. Biogas is one of the useful end products of the anaerobic digestion processes of organic waste where it is utilized to generate electricity. The most crucial and vital source for the generation of biogas in Malaysia is palm oil effluent (POME), livestock manure, and MSW (Ali et al. 2012; Mekhilef et al. 2014). The potential of electricity produced by the biogas is estimated to be 100 megawatts (MW) by 2015 (Shafie et al. 2011) with an energy reservoir of 410 MW by 2030 and of 360–400 MW by 2020 (Khor and Lalchand

Table 2.5 Production of biogas in Europe in 2015

European countries	Production of biogas mil m^3
Belgium	264
Bulgaria	23
Czech Republic	715
Denmark	177
Germany	9160
Estonia	15
Ireland	64
Greece	107
Spain	305
France	628
Croatia	42
Italy	2183
Cyprus	13
Latvia	102
Lithuania	27
Luxembourg	21
Hungary	93
Malta	2
Netherlands	381
Austria	350
Poland	267
Portugal	96
Romania	21
Slovenia	35
Slovakia	173
Finland	120
Sweden	195
UK	2627
Switzerland	128
Iceland	2
Norway	52
FYROM	6
Servia	7
Moldova	11
Ukraine	17
EU	18,207
Europe	18,429

Source: Scarlat et al. (2018)

2014). But as the Malaysia Sustainable Energy Development Authority (SEDA) stated to date, the cumulative installed capacity for biogas is only 6.48 MW and 6.36 MW (from landfill/agricultural waste) by 2015. Still, there is high Renewable

Energy potential from biogas, which could be garnered from MSW and this could be accomplished in a better way if there are more supporting policies (Bong et al. 2017).

2.5.1.3 Canary Islands

Biogas production from the animal manure in the farms of Canary Islands represents an additional energy source for heat and electricity production. 495.622 tons of manure per year is produced from all the farms on the island. Processing this manure for biogas production results in the overall 27.1 Mm^3 biogas potential per year with a comparable installed capacity of 6.8 MWe. If we consider 0.5 tons of manure production per day (the lowest limit for implementing biogas project), 546 farms raising various animal types have the potential for producing and utilizing their own biogas along with having electric powers ranging from 3 to 185 kWe. The biogas production has the capacity to inhibit GHG emissions which is equivalent to 55,745.1 tons of carbon dioxide solving both problems, the substitute for fossil fuels and appropriate management of animal manure.

2.5.2 Cost–Benefit Analysis of Biogas

2.5.2.1 Biogas Benefit

The cost–benefit analysis is the total value of biogas, a function of the net amount available, the conversion efficiency, and the value of fuel it replaces (House 2010). In addition to this, the fertilizer value of effluent is added as a benefit. The most usual practice for the disposal of MSW is landfilling. The treatment of organic solid wastes (accounting for about 70% of waste in the MSW) can divert a large part of the MSW from the landfill resulting in saving space and elongating the lifespan of the landfill. Besides, transport costs are also saved.

2.5.2.2 Environmental Benefits

The environmental aspect covers the advantages of an AD facility to the environment. The first and foremost being the reduction of the wood consumptions, which ends up in halt to the soil erosion conditions. The production of biogas plays a role in the global brawl counter to global warming. It acts as a substitute for natural gas and other fossil fuels polluting the environment. The use of the digestate reduces the consumption of artificial fertilizers, avoids carbon dioxide emissions, and deforestation is kept in check sustaining the capability of forests to act as carbon sinks (Kossmann et al. undated). All the benefits trail down to others as methane is reduced as a greenhouse gas by the contribution of anaerobic digestion.

2.6 Future Roadmap

Energy is an essential aspect for human and social development, but it is often produced at the expense of the environment and resulting in a plethora of environmental issues such as climate change (Khan et al. 2017). Biogas can help achieve

sustainable development at both ends providing the ability to cope with an energy crisis and casting a positive impact on the environment with maximum utilization of waste. However, some research work is required to overcome different gaps (Theuerl et al. 2019).

International technical standards for the consumption of upgraded biogas should be established. The development of international standards, with the participation of public and private sector stakeholders, is an effective way to deal with the energy crisis and sustainability issues (Awe et al. 2017). Some states and countries like California, Germany, and Sweden are already following the national standards for the utilization of the biogas. The maximum use of biogas is also an alternative to natural gas (Yentekakis and Goula 2017). Bureau of India standards—designed and authorized in 2013–accounts for the use of biogas in the transport system and stationary engines (Jana and Bhattacharya 2017).

The utilization of biogas has different aspects ranging from smaller to a broader spectrum. It facilitates in the heat production, fuel for fuel cells, a source of energy for various industries, upgrading of the natural grids, and production of proteins and chemicals. In future Biogas plants require the solution for the indigestible residues that hamper the efficiency of biogas plants (Meyer-Aurich et al. 2012). More research should be carried out to practice more efficient pre-treatment methods, cost-effective and less energy-consuming technologies for reduced methane leakages, and other environmental effects. It is required to determine the maximum sectors for the utilization of biogas. The fuel cell provides good opportunity for the use of the biogas; however, innovations in the market benefits and technological development are required to maximize the effectiveness of fuel cells (Kapoor et al. 2019). In China, the potential of straw biogas requires its application on a large scale. A more appropriate study is required to maintain the quality and quantity of the biogas (Yu et al. 2019).

In China, biogas production is restricted to rural areas and engineered gaps that reduce the efficiency and cause practical problems. The marketization of renewable energy is needed to promote in China and several other countries (Adams et al. 2015).

European countries use a high amount of agricultural and animal manure to produce biogas. China alters agricultural waste to biogas energy that not only copes with the energy crisis but also an excellent way to tackle waste generation problem. Bioenergy production from biomass has put up 55 EJ of total global energy source in 2012. The palm oil industry of Malaysia is using more than 36 biogas projects in Cleaner Development Mechanism (Aziz et al. 2019). This can be considered in two aspects as it helps the company to sell the carbon credits and reduce GHGs emissions.

Germany has advanced technology for biogas production. Transfer and transport of technology to developing countries would encourage them towards more efficient and competent processes of energy production. Renewable Energies Act first came into being in 2000 and provides incentives on the feed used for renewable energy. There was a rapid increase in the number of biogas plants from 1050 to 8292 from the year 2010 to 2012. Approximately 50% of the biogas generation in European

Union is contributed by Germany (Gao et al. 2019). Biogas production in the United States of America is the ultimate source of renewable energy among solar and wind energy production. (Shen et al. 2015). Sweden is a country that uses biogas for vehicle fuel and power generation. Biogas consumption provides rapidly mounting markets; the number of biogas upgraded vehicles has increased more than 70,000 vehicles (Holm-Nielsen et al. 2009).

Pakistan's forest cover is less than 5% (Rahman and Paatero 2012). Pakistan has completed massive afforestation projects of billion trees in 2017 but almost 4000 MW energy deficiency and wood among the only source in hilly areas inclines people toward the use of natural resources. Such efforts of restoration could only be fruitful with the utilization of renewable energy (Kharl and Xie 2017). Energy and transport are the two sectors that use more oil compared to others. National resources of crude oil are not enough to solve the congestion so huge amount of oil is being imported to meet energy requirements. For example, 4.98 million metric tons of oil were imported during July, 2016 (Khan et al. 2019). For the developing countries, like Pakistan, biogas production can play a major role in fixing the bottleneck of the energy crisis and boost the economy. Biogas plant can save 92,062 PKR by treating 10 m^3 of organic matter that is an excellent addition in the economy and can be used to provide better facilities to the mounting population of Pakistan (Ali et al. 2019). Biogas, from poultry waste only, can facilitate the country with 300 MWh/day of electricity that not only accommodates energy shortage but also is environment-friendly (Ali et al. 2016). Biogas is a sustainable way that ensures the safety of biological and ecological sources. In Pakistan fuelwood is a common source of domestic energy requirements whereas deforestation has reduced the availability of the fuelwood. So, it provides an alternative to the consumption of forest that has shortened up to 8.8%. Biogas has a good potential to produce 2.5 kWh of electrical energy are produced only by 1 cubic meter of biogas. Biogas has a positive relation with human health (Gao et al. 2019).

According to Rahman and Paatero (2012) 98.6 million buffalos and 174 million cows in India can produce 601.46 and 1044 metric tons of manure per year, respectively (Raheem et al. 2016). This high amount of animal manure can be used to produce biogas, as a result of this, India is the top third emitter of CO_2 with 2 GT CO_2 per year can cut down its share from the global emission (Wang and Zhou 2020).

The electrical energy produced by the biogas obtained from agricultural waste is considered as zero emitters of CO_2 because emitted CO_2 was part of the plant body (Hijazi et al. 2019). Bioenergy is an irreplaceable and integral substitute to the unquenchable demand for energy in future and reduces the harvesting of fossil fuel (Kapoor et al. 2019), that is 82% globally with the annual emission of 35 Gt CO_2 and trend of emissions is increasing in non-Annex countries (Perera 2018).

2.7 Conclusion

Biogas production can address problems of energy demand, SWM system, fossil fuel consumption, and global warming at the same time. It is of more importance to developing countries, like Pakistan and India, where energy deficiency leads to excess utilization of non-renewable natural resources and where animal manure production is more than enough, 1645.46 metric tons per year, to support the sustainable way of energy generation. There is a wide range of biogas utilization such as heat production, electricity generation, and domestic fuel consumption. Biogas has proved to be a sustainable approach to many countries, e.g. in 2016, Germany has fulfilled its 12.4% of energy requirements from biogas plants. Sweden uses biogas for vehicle fuel and power generation. The carbon-based fraction of the waste is used to produce biogas. Biogas production utilizes the diversity of substrates and thus a diversity of microorganisms to act upon them. However, microbial and biotechnological advancements can increase the efficiency and yield of biogas production in various ways: by bio-augmentation and addition of microorganisms. There is diversity in the organic waste which can be utilized for biogas production by different countries according to the availability of feedstock types. Food waste generation rates are higher in developing countries. Various technology options can be opted for biogas production depending upon the financial and human resource capacity of the nations. Technological transfer to developing countries will be helpful in inefficient biogas production. States and countries like California, Sweden, and Germany have established standards for the consumption of biogas. It is imperative to develop International Standards for the utilization of biogas sustainably. More research is needed to develop cost-effective technologies, pre-treatment methods, and reduction of environmental effects. There exists a dire need for the marketization of renewable energy in several countries to promote its production and consumption.

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3 Role of Thermophiles in Production of Aviation Biofuels: Fueling the Future

Latika Bhatia, Akhilesh K. Singh, and Anuj K. Chandel

Abstract

Presently, fossil fuels are consistent resource of energy for running ships, aircraft, trucks as well as automobiles that are key modes of transportation toward the efficient functioning of the society. However, rising greenhouse gas emissions and shrinking fossil fuel reserves lead to introduction of biofuels that offers many advantages to human race as well as the environment. Generally, the transport sector has shaped this potential toward adopting the unmet demands of biofuels from lignocellulosic materials. In current scenario, biofuel commercialization limitations are related to biomass processing and fermentation step. In this context, thermophiles owing to their exceptional benefits as well as the current advances of genetic systems enabling for metabolic engineering are perfect candidate toward filling the requirement of huge biofuel production from lignocellulosic material. These microorganisms have capability to withstand high temperatures resulting from heat produced in large-scale bioreactors. Remarkably, fermentation at such high temperature in large-scale bioreactors minimizes the chances of contamination. Thermophilic enzymes also have obtained considerable concentration owing to their capability to catalyze reactions of commercial significance at higher temperatures. Considerable research has been carried out using thermophilic microorganisms for bioethanol production but has not gained

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desired success at commercial operations. Genetic/metabolic engineering interventions could provide significant breakthrough in improvement of thermophilic ethanologens. This chapter focuses on the recent biotechnological advances that lead to the production of lignocellulosic-based aviation biofuel (bioethanol and biohydrogen) with special emphasis on thermophiles and their enzymes.

3.1 Introduction

Fossil fuels that have been highly praised globally for significant role into day's societal growth and development are nonrenewable as well as nonsustainable energy source. Furthermore, exploitation of these fossil fuels found to significantly contribute toward enhanced greenhouse gas (GHG) emissions like CO_2, etc., into the atmosphere as well as climate alteration. Consequently, the sustenance of environment, economy as well as the pace of growth and development in contemporary world can merely be attained through switching to the renewable energy resources (Chandel et al. 2020). All these concerns act as driving force for massive motivation in the direction of biofuel research owing to the shrinking of fossil fuel resources for huge commands of transportation together with enhanced discharge of GHGs. Also, governments of various countries like the USA, Italy, China, Germany, and India have invested substantial funds in the direction of biofuel research and also announced subsidies and incentives to vendors that manufacture biofuels (Mallick et al. 2016; Sharma and Singh 2016; Bhatia et al. 2018; Chandel et al. 2018).

Considering aforementioned facts, biomass-based transport fuels have received immense interest in the present global scenario as green renewable energy over fossil fuels with intention to expand plans to improve energy self-reliance, decrease importation of prices, as well as reinforce domestic agricultural progress. These biofuels not only reduce automobile discharges along with enhanced sustainability but also have been helpful in a shift to low-carbon fuels, which would lead to sustainability in transport segment. Exploitation of biofuels leads to substantial decrease of oil consumption as well as CO_2 emissions. Many industries like aviation, marine transport, and heavy freight direct themselves toward the exploitation of biofuels as low-carbon option to fossil fuel. Remarkably, among these industries, aviation industry alone will be accountable for rise in overall GHG releases in the range of 400–600% between 2010 and 2050. Thus, cost-effective mass production along with eco-friendly approach is all what aviation industry is looking for when it focuses on developing fuels (Lee and Mo 2011). As a result of this environmental threat, the aviation industry took accountability to diminish CO_2 emission and with this purpose, they flew their first profitable test flight by exploiting biofuels in 2008 (Araujo et al. 2017). Biofuel turns out to be a part of half of the jet fuel mixture of nearly twenty-two airlines in the mid of 2015, with the backing of which these airlines had completed >2000 passenger flights. In recent years, around 191 countries have directed themselves for the strategic control of aviation pollution through signing a treaty, thus developing a vast potential/scope toward nonstop

biofuel exploitation. A range of techniques have been developed for the production of sustainable fuels like biodiesel, bioethanol, biobutanol, and bio-aviation fuel with biomass as raw material. However, research on fabrication of bio-aviation fuel from renewable raw material is in vogue in current scenario. Hydrogenated esters as well as fatty acids processes were developed by UOP/Honeywell to elevate vegetable oils along with fats for sustainable jet/diesel fuels (Wang et al. 2018). Currently, biobutanol is produced from stock of corn using thermophilic *Clostridium* by green biologics. Substantial volumes of jet fuels, bioethanol and biodiesel that are intended to be used for civilian and military purposes, are commercially produced by corporations, such as Gevo, Joule Unlimited, and Solazyme. Reinstatement of crude oil used in the present infrastructure of petroleum is possible now. This is mediated by the use of a product named "Green Crude," which is a generation of Sapphire Energy (Coker 2016). There are many deciding factors particularly concerns for safety, performance of fuel, and cost-effectiveness, which together determines the replacement of fossil fuel with biofuel that is chemically identical to it.

3.2 Second-Generation Biofuel for Aviation Industry

In recent years, aircraft along with engine manufacturers, oil companies, governments as well as investigators have focused toward bio-jet fuel as a thrust area of research (Jimenez-Dıaz et al. 2017). Transportation fuels have shifted their focus from fossil fuels to biomass-based fuel owing to increasing fear of climatic changes along with anthropogenic changes like political instability in oil-opulent domains. In present context, bio-jet fuel is recognized as potential solution not only for reduction of GHG emissions from aviation company but also advocates energy salvation, where severe environmental problems along with the unsteadiness of oil bazaar are attracting the global concerns. Though approved for fabrication, many sources of bio-jet fuel still face hurdles in their commercialization and invasion into the market (Jimenez-Dıaz et al. 2017). One more reason for aviation industry to paying good attention toward bio-based alternative jet fuels is its growing concerns toward air traffic, as it is having impacts on environment. Alternative jet fuels support lower life-cycle petroleum usage along with GHG emissions (Han et al. 2017).

It is obvious that if we depend upon the use of first-generation feedstocks, it will lead to extensive changes in land and therefore adding more GHGs to atmosphere. Considering this, there is urgent need to switch over to next-generation biomass feedstocks that would result in no competition with food production. Apart from this, it is not dependent on fresh water for its cultivation. Fast growing plants that can be cultivated in wastelands with minimum farming techniques, would impose no threat for biodiversity, help local communities by providing socioeconomic assistance, and give rise to lesser carbon footprint are considered best suited for "second-generation" biofuels production. Biomass like jatropha, camelina, algae as well as halophytes are few examples that have potential to grow worldwide, due to their ability to tolerate conditions of desert and salt water. They are the best source of bio-derived oil. These

Table 3.1 Global warming potential (GWP) % reduction of various bio-jet fuels over petroleum-based jet (petro-jet) fuel

Bio-jet fuels/process	% GWP[a] (lower than petro-jet fuel) (%)	References
Oil seed-based jet fuels	41–70	Hileman et al. (2009)
Bio-jet fuel from corn stover pyrolysis	55–68	Hileman et al. (2009)
Fischer–Tropsch bio-jet fuel from biomass	81–89	Hileman et al. (2009)
Hydroprocessed and Fischer–Tropsch bio-jet fuels	74	Agusdinata et al. (2011)
Advanced fermentation of biomass	130–0.2	Staples et al. (2014)

[a]*GWP* global warming potential

biofuels are believed to have the potential to reduce CO_2 emission up to 80% over fossil fuels. In case of aviation industry, camelina feedstock has shown this reduction up to 84% (Lee and Mo 2011). Moreover, these requirements are also met by "drop-in" biofuels, which are synonyms of hydrocarbon biofuels. Presently, researchers involve in the production of bio-jet fuel and focus on various strategies like hydroprocessing of oil seeds, pyrolysis, gasification/Fischer–Tropsch, or progressed biomass fermentation. Drop-in bio-jet fuels share a similarity with ethanol, as both these fuels are potential enough to significantly bring down GHG releases. Table 3.1 shows the percentage global warming potential (GWP) reduction of various bio-jet fuels compared with petroleum-based jet (petro-jet) fuel (Hileman et al. 2009).

Second-generation biofuel contains lesser impurities such as sulfur, thereby aiding in greater reduction toward emission of soot and sulfur dioxide (Lee and Mo 2011). An outcome as a general consideration that has been derived from recent studies and research indicates ethanol to be a potential agent that can lower GHG liberations over petroleum-based fuels. Hydrogen is also considered as upcoming option to mitigate the problems of emission of particulate matter and NO_X. Moreover, CO_2 is not emitted at point of use, if engines are fueled with hydrogen. Due to these reasons, hydrogen (H_2) is viewed as substitute fuel for upcoming low-discharge aircraft (Lee and Mo 2011). Hence, in this context, there is urgent need to consider the role of various thermophilic microbes and their enzymes involved in production of liquid (ethanol) and gaseous (hydrogen) biofuels that are used in aviation. Thermophiles owing to their exceptional benefits as well as the current advances of genetic systems enabling for metabolic engineering are perfect candidate toward filling the requirement of huge biofuel production from lignocellulosic material. These organisms have the capability to withstand elevated temperatures that can result from heat produced in large-scale bioreactors (Chandel et al. 2012a). Remarkably, working at such high temperature in large-scale bioreactors leads to fewer chances of contamination. Thermophilic enzymes also have received considerable attention owing to their capability to catalyze reactions of commercial significance at higher temperatures.

3.3 Microbial Enzymology of Biofuel Production Including Thermophiles

Enzyme constitutes one of the vital bioproducts and finds its utility in many technological sectors like food, environment, and industry. Enzymes are finding newer and newer utilities/applications as biotechnology is advancing. Enzymes derived from filamentous fungi are commercially sound over the bacteria or yeast in terms of not only quality but quantity too (Bakri et al. 2003). Cellulase is efficiently synthesized by *Aspergillus niger* as well as *Tricoderma viride*, which are considered as safe organisms (Comacho and Aguilar 2003). These organisms are being used from a long time for production of extracellular enzymes, as these organisms possess potent protein secretion machinery that generates homologous and heterologous proteins. *A. niger* is a known organism to generate huge variety of enzymes, specially xylanases, hemicellulases, cellulases, and pectinases. With the aid of these enzymes, *A. niger* has potential to degrade plant cell material in its natural environment (de Vries 2003). Cost-effective sugars that are produced through enzymatic saccharification of cellulose can be fermented into biofuels and biochemicals (Kotchoniet al. 2003). Natural cellulose is made up of straight homopolysaccharide, where D-glucose residues connected with β-1,4-glucosidic bonds to that give rise to a polymeric chain. Cellobiose consists of two glucose units and is considered as smallest repetitive unit in cellulose. Cellobiose and glucose sugars are generated from cellulose when its β-bonds are hydrolyzed, thereby making cellulose as a valuable substrate for fabrication of value-added chemicals/products including biofuels (Chandel et al. 2012b). There are many microorganisms that produce cellulolytic enzymes. Prominent among them are bacteria and fungi that can degrade cellulose with the aid of their cellulolytic enzymes. These extracellular cellulases have extensive potential to solubilize crystalline cellulose (Mach and Zeilinger2003). The fungi with the same potential belong either to the white rot basidiomycetes, deuteromycetes, or ascomycetes group.

Thermophiles along with other wide variety of mesophiles, anaerobes, aerobes, bacteria, fungi, and actinomycetes are capable of producing cellulases (Rao et al. 2013). A complete complex of cellulases and that too of high yield is produced by fungi, which make them most studied organisms. *T. reesei*, *T. viride*, *P. pinophinum*, *P. chrysosporium*, *F. solani*, *T. emersonii*, *T. koningii*, and *R. oryzae* are aerobic fungi that are well characterized for their capacity to produce celluloses (Bhat and Bhat 1997; Murashimaet al. 2002). Interestingly, *C. thermophile*, *H. insolens*, *H. grisea*, *T. moidea*, *M. thermophila*, *T. emersonii*, and *T. aurantiacus* (Maheswariet al. 2002) are the prominent aerobic thermophilic fungi capable of producing cellulases. While, *N. frantalis*, *P. communis*, and *S. communis* are the prominent mesophilic fungi that also have potential to produce cellulases (Bhat and Bhat 1997). Microorganism secretes the cellulolytic enzymes into the medium. These enzymes are also associated with the outer surface of microorganisms that is capable of degrading cellulose. The enzymology of cellulose degradation is documented in several reviews (Kang et al. 1999; Lynd et al. 2003). Four types of enzymes are found to involve in the saccharification process of cellulosic biomass: (1) Endoglucanases (endo-1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4) randomly

hydrolyze internal 1,4-glucosidic linkages within the cellulosic chain of cellulose, leading to formation of gluco-oligosaccharides. Large numbers of reducing as well as nonreducing ends are formed in the oligosaccharides; (2) Exoglucanases (Exo-1,4-β-D-glucan-4-glucanohydrolase) cleave only external β-1,4-glucosidic bonds from nonreducing terminal of cellulose and also oligosaccharides and tear off glucose units; (3) Cellobiohydrolases (Exo-1,4-β-D-glucan-4-cellobiohydrolase EC 3.2.1.91) generate cellobiose units from nonreducing terminal of cellulose. The distinction between exoglucanases and cellobiohydrolases is always not clear, and (4) β-glucosidases (β-D-glucoside glucohydrolase EC 3.2.1.21) aids in breakdown of cellobiose and dextrins of low molecular weight to glucose. Organisms producing cellulases differ from each other in possessing different number of components of cellulases, as there are multiple enzyme components present in cellulases. There are two types of cellobiohydrolases, which are immunologically different from each other, these are CBH I and CBH II. *Trichoderma* spp., particularly *T. reesei*, is the known producer of these cellobiohydrolases and is known to secrete them in extracellular medium (Kubicek and Pentill 1998). Both of these cellobiohydrolases work in synergistic or cooperative way. Reducing and nonreducing ends are generated when endoglucanase works upon linear cellulose molecules. These ends are targeted by exoglucanase, which highlights more inner sites for endoglucanase binding. Cellobiose causes feedback inhibition of CBH. If somehow this cellobiose is converted into glucose with the help of β-glucosidase, this feedback inhibition is greatly reduced, and cellulolytic activity continues (Wood et al. 1995). Even synergistic interactions (exo–exo synergism) between isozymic forms of exoglucanase (CBH I as well as CBH II) occurred in solubilizing crystalline cellulose (Henrissat et al.1985).

3.4 Enzymatic Hydrolysis of Pretreated Biomass

The cellulose and hemicelluloses that are released once the pretreatment is over are required to be hydrolyzed to their monomeric constituents' sugars, viz., hexoses and pentoses. This step is mediated with the intervention of various enzymes like cellulases and hemicellulases. Cellulases lead to breakdown of cellulose, and hemicellulases do the same with hemicelluloses. The preliminary transformation of lignocellulose to sugars is a main congestion in the procedure of biofuel generation, and novel biotechnological unfolding is required to elevate their effectiveness, which would reduce the comprehensive rate of bioethanol manufacture (Bhatia et al. 2012). When we use enzymes, we do not have to face corrosion problems. Maintenance costs are lower and conditions of processing are mild, which together give higher output. If the hydrolysis is carried out enzymatically, this would make the bioethanol production economically feasible in a long run. There are many hurdles in biological translation of lignocellulosic residues to ethanol. Most prominent is the absence of most of the enzymes or their insufficient production that are required to completely degrade cellulose and hemicelluloses. Remarkably, thermostability of these enzymes is another barrier toward effective biotransformation of

lignocellulosic material into fermentable sugars. Moreover, recalcitrance nature of plant cell walls makes them resistant toward hydrolysis by microbes (fungal and bacterial strains) and their enzymes. These factors are the main issues, which have to be addressed so as to make bioethanol production inexpensive. Discovery of novel, potent varieties of thermostable biocatalyst that are able to bear extreme conditions stably is the current demand and has become the main motto of many researchers (Bhatia et al. 2012).

3.4.1 Fungal Extracellular Cellulases

There are many lignocellulosic materials that are presently being investigated for their enzymatic saccharification by cellulases. Main among these materials are agro-residues, forest residues, straw of rice and wheat, bagasse, etc. There are many microorganisms that can produce cellulases (also known as glucanases). These organisms can be bacteria or fungi, aerobic or anaerobic, and mesophile or thermophile. *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteroides*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* are the important cellulose-producing bacterial genera. *C. phytofermentans*, *C. thermocellum*, *C. hungatei*, and *C. papyrosolvens* are the anaerobic bacteria that potentially generate cellulases with elevated specific activity. *T. reesei* is the known producer of commercial glucanases (cellulases). *A. niger* is producer of β-D-glucosidase. *S. rolfsii*, *P. chrysosporium*, and various species of *Trichoderma*, *Aspergillus*, *Schizophyllum*, and *Penicillium* are known to generate cellulases (Bhatia et al. 2012).

Thermostable and acid tolerant enzymes are a choice for hydrolysis presently, and strategies adopted for pretreatment generally depend on acid and high temperature. Higher stability and specific activity are two important beneficial points of thermostable enzymes, which enhance their performance of hydrolysis. This would ultimately reduce the operation cost of hydrolysis as lower dose of enzymes is sufficient. The key producers of these thermostable enzymes are *T. emersonii*, *Chaetomium thermophilum*, and *Corynascus thermophilus*. The optimal temperature for growth of these organisms is 30–55 °C. Enzymes produced by these organisms can perform well at high temperature of 60 °C and can be used in industrial scales. *C. thermocellum*, *C. cellulovorans*, and *T. reesei* are among the best organisms that can proficiently degrade cellulose as well as xylan. Cellulosome complex is produced by *C. thermocellum* and *C. cellulovorans*, which is organized on their cell surface. This complex consists of cellulase and hemicellulase. Interestingly, extracellular secretions of *T. reesei* consist of three types of cellulases: (1) five endoglucanases (EG [EC 3.2.1.4]), (2) two cellobiohydrolases (CBH [EC 3.2.1.91]), and (3) two β-glucosidases (BGL [EC 3.2.1.21]) (Bhatia et al. 2012). Functions of these enzymes are mentioned in Sect. 3.5 of this chapter.

3.4.2 Fungal Hemicellulases

Hemicelluloses are heterogenous and hence need variety of enzymes for their complete hydrolysis. Over 70% structure of hemicelluloses is made up of xylan. β-1,4 bonds in xylan are the target sites of xylanases, hydrolyzing which it generates oligomers that are later broken down to xylose with the aid of β-xylosidase. Hemicellulose may also be comprised of other different components like mannan or arabinofuranosyl or arabinose in different composition, hence supplementary enzymes such as β-mannanases, arabinofuranosidases, or α-L-arabinases may also be required for hydrolysis of hemicelluloses. Hemicellulase belongs to glycoside hydrolases and to carbohydrate esterases, which breaks down ester bonds of acetate or ferulic acid side groups. Some fungi are the known producer of cocktail of huge quantities of extracellular cellulases as well as hemicellulases, and prominent among them are species of *Penicillium*, *Aspergillus*, and *T. emersonii*. Presence of cellulases along with hemicellulases or pectinases can efficiently enhance the magnitude of cellulose transformation (Bhatia et al. 2012).

3.4.3 Fungal Ligninases

Lignin is known to be the tough part of lignocellulosic material, but certain fungi have the inherited potential to degrade this tough part, by the virtue of their secreting enzymes termed ligninases. Among the most well-studied ligninase-producing organisms, *C. versicolor*, *P. chrysosporium*, and *T. versicolor* are the white-rot basidiomycetes, which can proficiently break down lignin. Fungal ligninases belong to two ligninolytic families: (1) phenol oxidase (laccase) and (2) peroxidases (lignin peroxidase [LiP] as well as manganese peroxidase [MnP]). Many fungi that are able to degrade lignin are found deficient in lignin peroxidases (LiPs). LiP is responsible of oxidation of nonphenolic part of lignin. Oxidative ligninolytic enzymes have to face the limitation that they cannot cross the cell wall. Size of these enzymes is responsible for it. Bacterial cellulosomes are well characterized compared to fungal ones, and hence it becomes mandatory that before enzymatic effect, low-molecular weight diffusible reactive oxidative substances start the changes to the lignin structure and hemicelluloses (Bhatia et al. 2012). Being an originator of contemporary heat-stable enzymes, thermophiles are of special interest. Traditional chemical processes are replaced or supplemented by a recent spawning of sturdy enzymes that are fitted to tolerate extreme circumstances in processes prevailing in industries. It is because of these properties of thermophilic enzymes, many thermophilic microorganisms are making their way toward biotechnological and commercial use (Mehta et al. 2016). Moreover, the risk of bacterial contamination is significantly reduced in the thermophilic fermentation due to inability of normal environmental contaminants to reproduce themselves. It can be said that the thermophilic fermentation can be best carried out even without pasteurization, which makes it efficient and a cost-effective operation. Moreover, thermophilic fermentation can well coordinate with simultaneous saccharification process (operating at 55 °C), thereby again

cutting down the operating cost involved in separate processes. Also, enzymes that are able to tolerate higher temperatures promote faster reactions, allow high solubility of the substrate, and also lower the viscosity of solution, thereby enhancing the solvent's mixability. Thermophiles possess many additional benefits such as wide spectrum of substrates, thereby allowing them to go for simultaneous degradation of both hexoses and pentoses; some thermophiles are inherently robust to break down complex carbohydrates. Use of thermophiles enables a one-step operation of enzymatic breakdown, fermentation, and distillation of formed ethanol (Xiao et al. 2012).

3.5 Habitat of Thermophiles

Thermophiles can potentially grow at temperatures of 55 °C or more than this. The minimum temperature necessary for their growth is usually around 45 °C, and they often have optimum temperature in the range of 55–65 °C. Majority of prokaryotes and some of the algae and fungi are thermophiles. Habitats like composts, self-heating haystacks, hot water lines, and hot springs are best suited for their propagation. These organisms possess enzymes that are stable at high temperature as well as machineries of protein synthesis are capable of functioning even at elevated temperature. These features make thermophiles different from mesophiles. Lipids present in membranes of thermophiles are highly saturated and possess elevated melting points. It is due to this property that the membranes of these organisms remain in their configuration at higher temperature. Hyperthermophiles cannot grow well below 55 °C, as their optimal temperature toward cultivation lies in the range of 80–113 °C. There are few hyperthermophiles like *Pyrococcus abyssi* and *Pyrodictium occultum*, which are able to exist in hot areas of the seafloor. Sulfide chimneys or "black smokers" are also a well-known habitat for thermophiles. The temperature of superheated vent water, which is rich in sulfide is about 350 °C. These microbes can grow and reproduce at 113 °C. Seawater does not boil until 460 °C, as the pressure found in these habitats is about 265 atm, which is responsible for keeping water at liquid state. Crenarchaeota are the thermophiles that are not only acidophiles but sulfur dependent too. Sulfur serves as an electron acceptor in anaerobic respiration or as an electron source by lithotrophs. Solfatara are the habitats where hot springs are found, which are sulfur enriched in Yellowstone National Park, Wyoming. Solfatara also include the waters surrounding areas of submarine volcanic activity. Solfatara are also fields of elevated temperature found within zones of volcanic activity, with high sulfur acidic soil, acidic hot springs, and boiling mud. Thermophiles can grow in soils rich in sulfur as well as in geothermally heated water. Almost all thermophiles are anaerobes.

The photosynthetic bacteria such as cyanobacteria, green, and purple bacteria are the examples of the thermophile marine microorganisms. The prominent bacterial domains that are thermophiles include *Actinobacteria* sp., *Bacillus* sp., *Clostridium* sp., *Desulfotomaculum* sp., *Thermus* sp., *Thiobacillus* sp., fermenting bacteria, spirochetes, and numerous other genera. The archaea domains such as *Pyrococcus* sp., *Sulfolobus* sp., *Thermococcus* sp., *Thermoplasma* sp., and methanogens are also

thermophiles (Dalmaso et al. 2015). Sulfur and hydrogen serve as the source of electron for lithotrophs. As most of the hyperthermophiles are strictly anaerobic, organotrophic, and lithotrophic, they are able to survive in solfataric fields of terrestrial ecosystem as well as deep reservoir of oils. Hyperthermophiles are also adapted to survive in these places as they are neutrophilic and slightly acidophilic. One of the archaea that has been isolated from geothermally heated sea floors is *Pyrodictium*. The optimal temperature for growth of this microorganism is 105 °C, whereas the minimum and maximum temperature range is 82 °C and 110 °C, respectively (DeCastro et al. 2016).

Biotopes are biotic and abiotic factors that regulate the growth of all living organisms. Shallow submarine hydrothermal systems and abyssal hot vent systems are also the places, where thermophiles exists. These systems are collectively known as black smokers, and the range of temperature prevailing in these systems is about 270–380 °C. The black smokers are mineral rich hot waters that make cloud of precipitated material on mixing with seawater. The pH of the smoldering coal refuse piles (another biotype) is acidic and has reservoirs of geothermally heated soil. Thermophiles are isolated from these habitats too (Manoharan et al. 2015).

3.6 Thermophilic Enzymes: Potential Platform in Bioethanol Production

At many stages, biofuel production involves elevated temperature and acidic pH. Extremophillic microorganisms have the potential to grow in extreme conditions with the active physiological state. It is because of these potentials that extremophiles have replaced mesophiles that were used in conventional methods. For instance, *Thermoanaerobacterium saccharolyticum* has potential to consume xylose (pentose sugars from hemicellulose fraction of plant cell wall). Engineered form of *T. Saccharolyticum* has shown great assurance in bringing forth huge quantities of ethanol and reducing other side reactions or products (Coker 2016).

The enzymes of thermophiles are of great interest. Owing to the thermal stability of the enzymes, hyperthermophiles are receiving special attention as the implications of this discovery are many. In the coming years, there is a feasibility to design enzymes, which are capable to perform at higher temperatures. These enzymes play important role in the manufacture of methane, leaching of metals and its recovery, and for usage in systems, where enzymes get immobilized. With the aid of latest thermostable enzymes discovered, chemical syntheses are now possible directly in which the compounds can be selectively and stereochemically modified. This makes modern biological sciences a thrilling and elaborated area, where microbiologists and biotechnologists can contribute significantly (Arora and Bell 2012). There are a variety of hydrolases that are produced by thermophiles. Prominent among them are lipases that degrade lipids, peptidase that degrades peptide bonds of proteins, amylase that degrades starch, and cellulose, as previously discussed, degrades cellulose. These enzymes, due to their immense utility, are researched extensively. Enzymes generated from thermophiles have potential to perform in adverse

physiochemical conditions, and these enzymes find their utility in many industries such as the biofuel, pharmaceutical, fine chemicals, and food (Singh 2012). There are many other industries that involve the usage of extremozymes of thermophiles. In this context, paper and bleaching industry and first- and second-generation biofuels industries are some important examples (Morozkinaet al.2010). Starch hydrolysis rate gets enhanced at an elevated temperature by the involvement of amylase that has thermostability. The chances of contamination are, thereby, highly diminished.

There are many enzymes that not only possess thermostability but can efficiently perform in wide range of pH. These enzymes are sold by different companies by many brand names. One such example is Fuelzyme® (Verenium Corporation, San Diego, CA). It is an amylase of alpha type and its source is a thermophile *Thermococcus* sp. whose habitat is a deep-sea hydrothermal vent, from where it is isolated. Mash liquefaction is mediated by Fuelzyme® during production of ethanol. This process releases dextrins and oligosaccharides, which possess low molecular weight and get easily solubilized. pH range and temperature at which Fuelzyme® works potentially is 4.0–6.5 and 110 °C, respectively (Karan et al. 2012). However, Fuelzyme® and Spezyme® (DuPont-Genencor Science) are exceptionally meant for generation of biofuel. Processing of industrial starch becomes faster when a cocktail of theses branded enzymes is used. Amylases isolated from *Bacillus* sp. are found perfect for applications during downstream process (Gabani and Singh 2013).

For an efficient bioconversion of a substrate to viable bioethanol production, it is mandatory to utilize the entire variety of carbohydrate components present in lignocellulosic material. D-xylose is the component of xylan. Many organisms are reported to produce ethanol, both from D-xylose and xylan. Important ethanol producers from these substrates are *T. ethanolicus*, *C. thermocellum*, *C. thermohydrosulfuricum* (reclassified as *Thermoanaerobacter thermohydrosulfuricus*), *T. brockii* (reclassified as *Thermoanaerobacter brockii*), *C. thermosaccharolyticum* (reclassified as *Thermoanaerobacterium thermosaccharolyticum*), and *T. saccharolyticum* B6A. These organisms are anaerobic thermophiles that have the optimum growth temperature of 70–80 °C. These organisms have been explored from various habitats, main among which are hot springs, paper pulp mills, and brewery wastewater. These thermophiles have potential to covert monomers of hemicelluloses like xylose, arabinose, mannose, and galactose to ethanol, where the yield and productivity are found to be satisfactory (Scully and Orlygsson 2015). Likewise, *Kluyveromyces marxianus* 6556 is also a thermotolerant strain that has the capability to undergo simultaneous saccharification and fermentation (SSF) of lignocellulosic agricultural residues. These species can grow potentially well around 40 °C. They can ferment the cocktail of variety of sugars like glucose, xylose, mannose, and galactose. Some strains of this organism are physiologically active at 52 °C, where they can speedily get replicated and have high growth rate (Rajoka et al. 2003). Xylose can get converted to ethanol under SSF at elevated temperature, utilizing *K. marxianus* (Fonseca et al. 2007). *K. marxianus* possesses genes that encode for many thermotolerant enzymes, viz. cellobiohydrolase, endoglucanase, and β-glucosidase (Hong et al. 2007). When *K.*

marxianus strain was modified to exhibit endoglucanase II of *T. reesei* and β-glucosidase of *Aspergillus aculeatus* on the cell surface, this organism could effectively produce 4.24 g/l of ethanol by converting 10 g/l of a cellulosic β-glucan at 48 °C within 12 h (Yanase et al. 2010). Fermentation is also mediated by anaerobic bacteria that are thermo-active. Icelandic hot springs are a hub of some thermophiles that can produce significant amount of ethanol from lignocellulolytic hydrolysates. Combination of cellobiohydrolase (of *T. reesei*) and endoglucanase (of *Acidothermus cellulolyticus*) enhance the yield of saccharification (Turner et al. 2007). In addition, *Thermoanaerobacter ethanolicus* or *Thermoanaerobacterium saccharolyticum* and *C. thermocellum* are other important thermophiles, which possess immense potential to produce ethanol. These thermophiles are able to manifest this production by the virtue of their bifunctional acetaldehyde-CoA/alcohol dehydrogenase that can form bioethanol from acetyl-coA. *C. saccharolyticus*, *T. tengcongensis*, and *P. furiosus* are also thermophiles that do not possess this enzyme/ system, due to which their ethanol production capacity is low (Bielen et al. 2013).

3.7 Role of Thermophiles and Their Enzymes in the Production of Hydrogen Fuel

There are many reasons to consider hydrogen gas (H_2) as a substitute for conventional fossil fuels. One of the imperative reasons for this substitution is that during the oxidation of hydrogen only H_2O is released, whereas with carbon-based biofuel it is CO_2. Second major reason for this substitution is that H_2 fuel cells are comparatively more energy efficient. Dark fermentation is the term given to an anaerobic fermentation process in which renewable feedstocks are used for the production of biohydrogen. This process is different from photofermentative hydrogen production. CO_2 would not get released during this process and the production of biohydrogen takes place in controlled manner, thereby preventing the environmental dissemination of CO_2.

Thermophiles like *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii* are some of the thermophiles that have aided in the recent development of larger-scale microorganism-based systems employed in hydrogen production that relied traditionally on chemical/catalyst procedure (Das and Veziroglu 2001; de Vrije et al. 2002). *C. saccharolyticus* is considered to be an exceptional contestant for H_2 production biologically as it can produce 4 mol hydrogen per mol hexose that approaches the theoretical limit for dark fermentation. It is Gram-positive, anaerobic, thermophilic cellulolytic bacterium that has the potential to ferment a wide-range variety of monosaccharides, disaccharides, and polysaccharides including acetate. There are also copious utilizations for extremophiles in the hydrogen production through anaerobic fermentation and hydrogenases. Strains of *Caldicellulosiruptor*, *Thermoanaerobacterium*, *Pyrococcus*, and *Aeropyrum* possess enormous caliber (Coker 2016). *Caldicellulosiruptor saccharolyticus* that was isolated in mid-eighties is an excellent source of thermoactive glycoside hydrolases due to which it is not

only an excellent organism able to degrade polysaccharide like cellulose but also an excellent candidate for production of biohydrogen from renewable biomass (de Vries 2003). Thermophilic conditions are favorable for degradation of plant biomass and formation of hydrogen biologically. It is also a thermodynamic consideration that elevated temperature is best suited for the formation of hydrogen (Kengen et al. 2009). Because of this reason, (hyper)thermophiles can yield higher hydrogen, which reaches the theoretical limit of 4 mol H_2 per mol of hexose over mesophiles (Verhaart et al. 2010). The theoretical yield involves the pure catabolic constituent of glucose transformation (Bielenet al. 2013). The bacterium *C. saccharolyticus* has an optimum growth temperature of 70 °C. This organism is a bank of varieties of endo- and exo-glycoside hydrolases, which aids in degradation and growth on a diversity of biomass substrates that contains cellulose and hemicellulose. These endo- and exo-glycoside hydrolases (GH) have potential to hydrolyze the glycosidic linkages of huge varieties of macromolecules like β-glucans (starch, pullulan as well as cellulose), xylan, and hetero-polysaccharides (hemicelluloses and pectin) (Albertson et al. 1997). This feature makes *C. saccharolyticus* different from *Clostridium* species that utilize cellulosome-like structures for the disintegration of recalcitrant polysaccharides of plants. *C. saccharolyticus* prefers substrates in broad range, due to the presence of great variety of transport system found in the genome. The efficacious breakdown of recalcitrant plant polysaccharides into mono-, di-, or oligo-saccharides is also feasible by the virtue of the fact that few of the glycosidases have multidomains like glycoside hydrolase domains as well as carbon-binding modules. This organism can co-utilize hexoses as well as pentoses without showing any symptoms of carbon catabolite repression. This characteristic makes *C. saccharolyticus* suitable for any consolidated bioprocess (Willquist et al. 2010). In addition, there are various enzymes of *Caldicellulosiruptor* species, like β-glucosidase (BglA), β-xylosidase, β-1,4-xylanase, and a type I pullulanase that have been successfully cloned and characterized (Schofield and Daniel 1993). *Caldicellulosiruptor* species and *Thermoanaerobacter* species are known to degrade crystalline cellulose with the aid of their free-acting primary cellulases. Overall, economic feasibility of biohydrogen production from microbe including thermophiles needs substantial improvements in consumption rates of substrate along with hydrogen yield (Bielen et al. 2013).

3.8 Conclusion

In the current scenario, the microbial-mediated fabrication of second-generation biofuels using lignocelluloses is much more advantageous over first-generation biofuels. Extensive investigations have been carried out in the area of biofuel by focusing on microorganisms that depict growth in mesophilic temperature range, i.e., 25–37 °C. However, such mesophilic microbes have no ability to exploit lignocelluloses straightforwardly. In this context, thermophiles have been considered as promising microbes toward biofuels production as they not only break down the lignocelluloses effectively (by consolidated bioprocessing) and reduce the

possibility of contamination but also offer various thermostable enzymes and genes toward metabolic engineering. Remarkably, there is no report available so far in which a high-yielding biofuel (like ethanol) production route of thermophile has been introduced to a nonethanol forming/low-ethanol forming microorganism for high bioethanol production. Such nonavailability of report is due to the short of knowledge concerning to involved thermophilic enzymes. Moreover, the cost of thermophilic enzymes is a chief obstacle in manifesting their vast potential for various applications. It is certain that in coming years, there will be a continuous and elaborated demand for thermostable biocatalysts. Although the current research on thermophilic microbes concerning biofuels production is still at very initial stage, the contemporary biotechnological approaches like genetic and metabolic engineering of hyperthermophiles are quite promising to overcome limitations of thermophile-based biofuel production in the near future.

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Bacterial-Mediated Depolymerization and Degradation of Lignin

4

Madan Kumar, Raj Morya, Asmita Gupta, Vivek Kumar, and I. S. Thakur

Abstract

Today, more than two-thirds of global energy supplies come from fossil fuels. However, dwindling reserve of fossil fuels, precarious global oil market, energy geopolitics, and associated environmental impacts due to fossil fuel burning have compelled almost all the nations of the world to search for clean and renewable alternatives for energy, fuel, and chemicals. Among the renewable energy resources, biomass has been projected as a promising source of future alternative to substitute crude oil-based petro-refinery. Lignin degradation and its conversion into fuel and chemicals is a major unresolved problem in a lignocellulosic biorefinery. Lignin valorization is in nascent stage despite being a high priority in second-generation biofuel programs. Lignin valorization can be done by physical, chemical, physicochemical, and biological routes. This chapter focuses on biological route for bacterial-mediated lignin depolymerization and degradation. The importance of lignin utilization, its synthesis, major sources of its generation, and its structure is discussed. Bacterial strains, enzymes, and pathways involved in lignin degradation have also been elucidated. Furthermore, the role of genomics and proteomics in bacterial lignin degradation has also been discussed.

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4.1 Introduction

Lignocellulosic biomass is a carbon-neutral or low-carbon, easy available, and renewable feedstock available for the production of fuel and chemicals. Biomass is currently the single, largest source of renewable energy worldwide, providing 10% (50 EJ) of the global primary energy supply (IEA 2016). Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin. For the production of second-generation biofuels and chemicals, these components need to be separated into the individual component and must be fully utilized to make the lignocellulosic biorefinery environmentally, socially, and economically feasible (Sims et al. 2010; Singhvi et al. 2014). The lignocellulosic biomass-based research mainly focuses on polysaccharides component of biomass, and lignin is discarded as waste with very limited usage. Therefore, the challenges associated with the success of second-generation biomass-based biorefinery must be addressed (Singhvi et al. 2014). The recent focus on the concept of lignocellulosic biorefinery, i.e., utilization of biomass and byproducts leading to minimal or zero waste generation, can overcome the problems associated with biofuel production at commercial scale. Lignin is very less explored, and the advancement in approaches of lignin valorization into valuable bioproducts is essential for overall economic viability and sustainability of lignocellulosic biorefinery (de Jong et al. 2012; Ragauskas et al. 2014).

Lignin is recalcitrant to degradation due to its complex and heterogeneous structure. Its depolymerization is being done by chemical, thermochemical, and biological processes for conversion into fuel and chemicals. Compared to the chemical processes, the use of microorganism or enzymes for lignin depolymerization is less energy intense, cost effective, eco-friendly, and works at ambient temperature with fewer inhibitors' generation (Zhu et al. 2017). The discovery of new microbial strains and understanding their enzyme system that is responsible for lignin degradation will help in lignin depolymerization and its conversion into fuel and chemicals (Kumar et al. 2017).

4.2 Lignocellulosic Biomass and Its Composition

Lignocellulose is the main constituent of plant cell wall and mainly refers to the dry matter of plant biomass. Lignocellulosic biomass is a complex matrix composed of cellulose, hemicellulose, lignin polymers, and a small amount of proteins, extractives, and minerals (Moon et al. 2011; Menon and Rao 2012; Singh et al. 2017). The structure and composition of lignocellulosic biomass are represented in Fig. 4.1. Two-thirds of lignocellulosic biomass are comprised of cellulose and hemicellulose polysaccharides that are made of hexose (C6) and pentose (C5) sugars. These polymers organize themselves into a complex three-dimensional structure. Their organization is nonuniform with a varied composition of cellulose, hemicellulose, and lignin depending on the types of lignocellulosic biomass (Gírio et al. 2010; Scheller and Ulvskov 2010; Menon and Rao 2012). The presence of

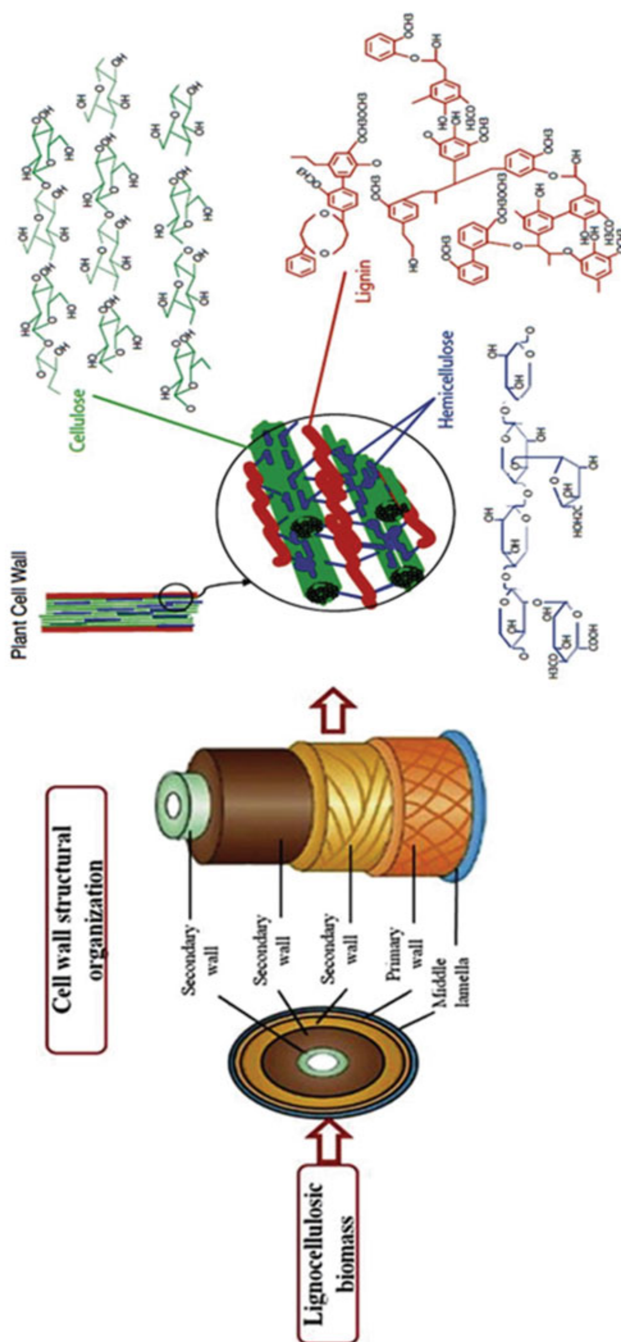

Fig. 4.1 Structural organization and composition of lignocellulosic biomass (adapted from Menon and Rao 2012; microbewiki)

Table 4.1 Composition of characteristic lignocellulosic feedstock (adapted from Harmsen et al. 2010; Menon and Rao 2012)

Composition of biomass (% dry wt.)			
Sources	Cellulose	Hemicellulose	Lignin
Sugarcane bagasse	25–45	28–32	15–25
Corn stover	35.1–39.5	20.7–24.6	11.0–19.1
Rice straw	29.2–34.7	23–25.9	17–19
Wheat straw	35–39	22–30	12–16
Grasses	25–40	25–40	10–30
Switch grass	35–40	25–30	15–20
Eucalyptus	45–51	11–18	29
Poplar wood	45–51	25–28	10–21
Pine	42–49	13–25	23–29
Softwood stem	45–50	24–40	18–25
Water hyacinth	18.2–22.1	48.7–50.1	3.5–5.4
Bamboo	49–50	18–20	23
Cotton stalk	31	11	30

individual lignocellulosic components in different biomass has been shown in Table 4.1.

4.2.1 Lignin

The term lignin was coined by a French chemist and botanist Anselme Payen in 1838 and was chemically defined by Schulze in 1885 (Doherty et al. 2011; Lange et al. 2013; Feofilova and Mysyakina 2016). Lignin encrusts the cellulose and hemicellulose components of plant cell wall and provides impermeability, mechanical strength, and rigidity to the plant cell wall. Lignin provides defense barrier and protects the plant against microbial attack. The distribution and concentration of lignin vary in plant cell wall and between different species (Lange et al. 2013). Lignin biosynthesis in plants occurs by phenylpropanoid pathway. Lignin monomer synthesis starts with deamination of phenylalanine to form cinnamic acid through a series of enzymatic reactions catalyzed by various enzymes (Bonawitz and Chapple 2010). The aromatic ring of cinnamic acid undergoes a series of reactions such as hydroxylation, *O*-methylation, and side chain reduction from acid to alcohol, to form three lignin monomers or monolignols: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. After synthesis, the monolignols are transported with the help of ABC transporter to apoplast. The monolignols are polymerized by radical–radical coupling generated by various oxidoreductases to form ether and C-C bonds (Bonawitz and Chapple 2010; Guerriero et al. 2016).

These three phenylpropane units (*p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) after polymerization get incorporated into lignin polymer as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units (Bonawitz and Chapple

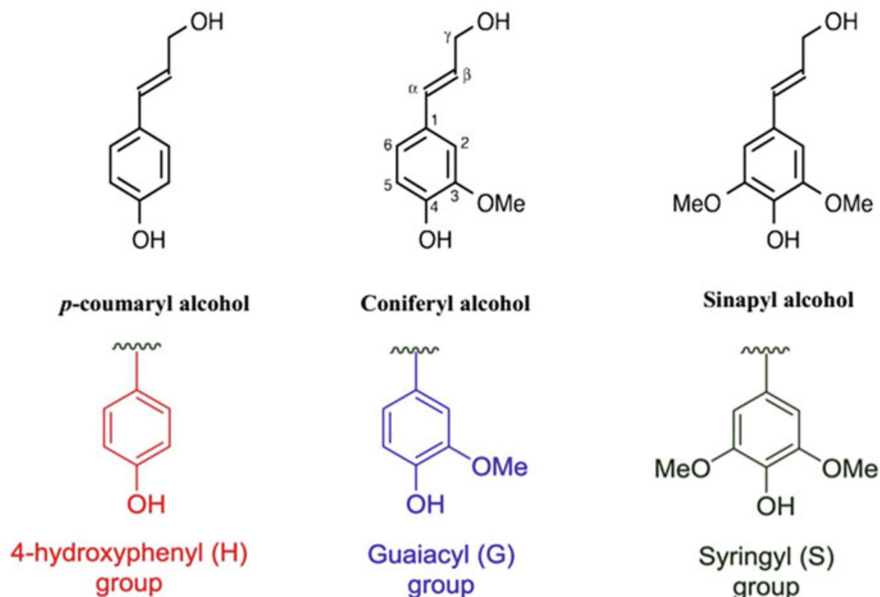

Fig. 4.2 Lignin monomers and their polymerization units in natural lignin polymer

2010; Doherty et al. 2011). Lignin monomer and its polymerization units in lignin polymer are shown in Fig. 4.2. The composition of H, G, and S units differs due to different bonding tendencies in lignin and varies between different species and within tissues of the same plant. The distributions are as follows: angiosperm mostly contains G and S, gymnosperm mainly contains G units, monocot relatively contains more H, coniferous wood mainly contains G followed by Sand H units, deciduous trees contain equal amount of G and S, and grasses contain all three units (Bonawitz and Chapple 2010; Feofilova and Mysyakina 2016).There can be other natural lignin monomers derived from phenolics other than familiar monolignols such as 5-hydroxyconiferyl alcohol, hydroxycinnamate esters, hydroxycinnamaldehydes, dihydrocinnamyl alcohol, hydroxybenzaldehydes, etc. (Ralph et al. 2004).

4.2.2 Sources of Lignin

Lignocellulosic biomass needs to be pretreated to separate it into its valuable individual components. The basic aim of pretreatment is to improve the biodegradability of cellulose by solubilizing hemicellulose and lignin and cellulosic pulp with reduced crystallinity and lesser degree of polymerization (Harmsen et al. 2010; Menon and Rao 2012; Gomes et al. 2014). Pretreatment methods can be divided into physical, chemical, biological and also their combination to make them more accessible for chemical or enzymatic applications. Selection of pretreatment method depends on feedstock, economic, and environmental impact assessment and

biological process to be applied (Doherty et al. 2011; Menon and Rao 2012; Lange et al. 2013). An effective pretreatment method should be cost effective, require less energy input, catalyst recovery, and recycle, avoid inhibitors' formation, and recovery of lignin and hemicellulose fraction as well as treatment of waste. Chemical pretreatments are most studied processes, and these are generally used in pulp and paper industry. Pretreatment is a prerequisite for feasibility of processes based on plant polysaccharides. Delignification is essential in pulp and paper mill industry and for biofuel production. Pulp and paper and plant polysaccharide-based industries are the major sources of lignin. The major types of lignin generated through different pretreatment processes applied in industries are Kraft lignin, lignosulphonates, soda lignin, and organosolv lignin.

4.2.3 Lignin Structure and Characterization

The exact structure of natural lignin is still unknown, but with the advancement in analytical technique some linkages commonly found in lignin have been revealed. The linkages are formed between oligomer–monomer, oligomer–oligomer, and monomer–monomer coupling reactions (Ralph et al. 2004). There are various interunit linkages observed in lignin polymer as shown in Fig. 4.3.

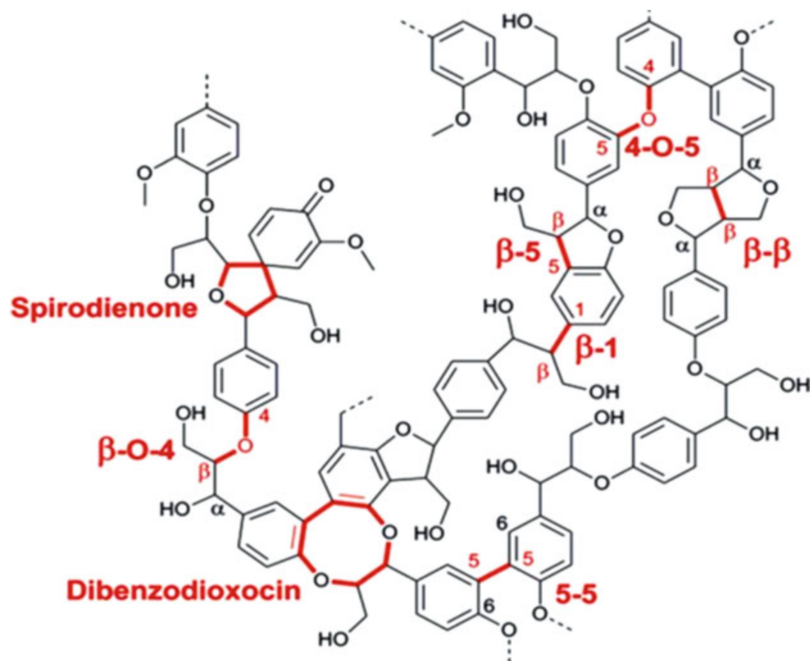

Fig. 4.3 Common interunit linkages found in highly complex three dimensional lignin polymer (Longe et al. 2016)

Table 4.2 Common interunit linkages and their composition found in natural lignin (Chakar and Ragauskas 2004; Munk et al. 2015)

Bond name	Linkage	Hardwood	Softwood	Grasses
Phenylpropane β-aryl ether	β-O-4′	45–50%	60–80%	69–94%
Phenylcoumaran	β-5′ and α-O-4	9–13%	3–11%	11–14%
Pinoresinol	β–β′	2–6%	3–12%	4–15%
1,2-Diaryl propane	β-1′	1–9%	1–7%	Low
Diaryl ether	4-O-5′	3.5–8%	6–9%	–
Biphenyl	5–5′	19–27%	3–9%	–
Dibenzodioxocine	α-β-O-4-4′ and 5-5′	5–7%	0–2%	3–4%
Spirodienone	Tetrahydrofuran aryl linkage	2–3%	3–5%	3–5%

The most abundant coupling is favored at β-carbon of one monolignol with hydroxyl group of the other to form β-O-4-aryl ether linkage. This is the major linkage observed in case of lignin structure. The others are α-aryl ether (α-O-4), phenylcoumaran (β-5), diaryl ether (4-O-5), biphenyl and dibenzodioxocin (5-5), spirodienone (β-1), and pinoresinols (β-β). The interunit linkages present in lignocellulose have been shown in Table 4.2. Coupling at position 4 and 5 is preferentially formed by dilignols and higher oligomers (Lange et al. 2013; Ralph et al. 2004; Chakar and Ragauskas 2004; Lupoi et al. 2015).

4.3 Lignin Degradation by Bacteria

Lignin is mainly degraded by fungi and bacteria in the natural environment. Fungi, especially white rot fungi, have been studied extensively for degradation of lignin by producing various ligninolytic enzymes (Bugg et al. 2011a; Kumar et al. 2015). The fungal biocatalysts for lignin degradation have not been commercialized till date due to genome complexity and information processing and difficulty in recombinant protein expression. Compared to fungi, the bacterial genome is small and easy to manipulate and recombinant proteins can be easily expressed and produced at a large scale (Bugg et al. 2011a; Kumar et al. 2015). So the focus again has been shifted to identification and characterization of novel bacterial strains and their enzymes responsible for lignin degradation. Recently, various new bacterial strains have been reported with lignin-degrading potential (Priyadarshinee et al. 2016). According to a recent review, 22 actinobacteria, 10 alpha proteobacteria, beta proteobacteria, 11 gamma proteobacteria, one delta proteobacteria, bacteriodes, and archaea, each were reported to be having lignin-degrading genotypes and phenotypes (Tian et al. 2014). Various bacterial enzymes such as β-esterases, DyP-type peroxidase, laccases, and various other oxidative enzymes responsible for lignin degradation have been recently reported (de Gonzalo et al. 2016).

Table 4.3 Showing some of the recently reported lignin-degrading bacterial strains (adapted from Longe et al. 2016; Priyadarshinee et al. 2016)

Bacterial strain	Substrate	Lignin reduction (%)	Days
Pandoraea sp. ISTKB	Kraft lignin	50.2	7
Pandoraea sp. B-6	Kraft lignin	38.2	7
Sphingobacterium sp.	Lignosulfonate	31	3
Novosphingobium sp. B-7	Kraft lignin	38.2	7
Planococcus sp.	Kraft lignin	55	2
Bacillus sp. CS-1	Alkali lignin	99.5	3
Comamonas sp. B-9	Kraft lignin	45	7
Cupriavidus basilensis B-8	Kraft lignin	31.3	7
Pandoraea sp. ISTKB	Sugarcane bagasse	10.4	20
Bacillus pumilus	Kraft lignin	50	18
Bacillus atrophaeus	Kraft lignin	70	18
Bacillus sp.	Alkali lignin	40 and 80	1 and 2
E. coli from beef cattle rumen	Maize stover	36.8	4

Recently, various bacterial strains have been reported for lignin degradation. Bacterial strains responsible for lignin degradation can be applied in valorization of biomass. Lignin-degrading bacterial strains having cellulose-free xylanase can be an excellent choice for pretreatment. The bacteria responsible for lignin degradation can be found in diverse environments such as soil, digestive system of herbivores, wood-eating insects, effluents from paper industry, sludge, etc. (Brown and Chang 2014; Tian et al. 2014). The bacterial strains recently reported for lignin degradation have been shown in Table 4.3. Advancement in genomics, transcriptomics, and proteomics completely revolutionized the understanding of microbial lignin degradation. Next-generation sequencing (NGS) technology resulted in complete genome sequence of several new microbes that will further enhance our understanding related to lignin degradation (Baldrian and López-Mondéjar 2014; Kameshwar and Qin 2016). The NGS (genomics and transcriptomics) along with proteomics provided various detailed information related to expression of proteins and characterization of new enzymes responsible for lignin utilization (DeAngelis et al. 2013; Lin et al. 2016; Zhu et al. 2017) Discovery of new microbes and further advancement and affordability in these technologies will enhance our knowledge in the near future.

4.4 Bacterial Peripheral Pathways for Lignin Degradation

Lignin is a complex polymer, and the products obtained after its depolymerization are highly heterogeneous, and therefore various pathways are involved in degradation of lignin. The bacterial enzymes have been characterized for lignin degradation, but degradation of lignin inside the cell is not clearly understood. Thorough understanding of catabolic pathways is very important for biotechnological application of

lignin-degrading microbes in lignocellulosic biorefinery. Some prominent pathways have been discussed.

4.4.1 β-Aryl Ether Degradation Pathway

β-aryl ether linkage is the most predominant linkages (50–70%) in lignin; therefore, cleavage of β-aryl ether bond is crucial for lignin biodegradation. The cleavage of ether bond leads to formation of various industrially important aromatic compounds. *Sphingobium* sp. SYK-6 has been extensively studied on various lignin model compounds for the degradation of β-aryl ether bond. Lig EFG gene cluster enzymes, lignin peroxidase, and β-aryl-OH elimination followed by decarboxylation, vanillate dehydrogenase, and demethylation mechanism has been reported for β-aryl ether metabolism (Masai et al. 2007; Bugg et al. 2011b). Lig EFG has been discussed in the enzyme section. The degradation of β-aryl ether bond has also been studied in *Rhodococcus jostii* RHA1, *Pseudomonas acidovorans*, *Pseudomonas putida*, *Pseudomonas* sp. HR199, *Novosphingobium*, etc. (Masai et al. 2007; Bugg et al. 2011b; Chen and Wan 2017).

4.4.1.1 Biphenyl Degradation Pathways

Biphenyl linkage is the second most abundant linkage (10%) found in lignin after β-aryl ether. Biphenyl is a major environmental pollutant and affects human health. The degradation pathway has been extensively studied in bacteria. Degradation of biphenyls has been studied in genus *Pseudomonas*, *Ralstonia*, *Burkholderia*, *Comamonas*, *Achromobacter*, *Rhodococcus*, *Acinetobacter*, and *Bacillus*. The reaction is initiated by a biphenyl 2, 3-dioxygenase of Rieske nonheme iron oxygenases family. Study on model compounds by *S. paucimobilis* SYK-6 suggested O-demthylation reaction followed by extradiol ring cleavage by dioxygenase and finally degraded by β-KAP pathway into acetyl-coA and succinyl-CoA (Masai et al. 2007).

4.4.1.2 Ferulate, Diarylpropane, Phenylcoumarane, and Pinoresinol Catabolic Pathways

Ferulic acid is attached by ester linkage to hemicellulose, and their degradation is carried out by esterases. Ferulate esterases have been identified in several bacteria. There are two types of pathway reported for degradation of ferulate. In one pathway, side chain cleavage occurs to eliminate two carbons from ferulate by two enzymes (a feruloyl-CoA synthetase, feruloyl-CoA hydratase/lyase FerB). The enzymes have been reported in *P. putida* WCS358, *Amycolatopsis* sp. HR167, *Pseudomonas* sp. HR199, *Pseudomonas fluorescens*, *Pseudomonas putida*, etc. Another pathway is the release of one carbon by nonoxidative decarboxylation of ferulate side chain. This pathway has been identified in *Bacillus* sp. BP-7 and *Enterobacter* sp. Px6-4 (Masai et al. 2007; Bugg et al. 2011b). This degradation of diarylpropane has been studied in *Pseudomonas paucimobilis* TMY1009. The enzyme responsible for diarylpropane degradation has been characterized while growing this strain on

diarylpropane model compounds, but the gene has not been identified. The product obtained is lignostilbene, and the enzyme (lignostilbenedioxygenase) responsible for its degradation into vanillin has been reported (Bugg et al. 2011b). The degradation of phenylcoumarane and pinoresinol has been studied with model compounds in *S. paucimobilis* SYK-6, but the genes responsible for degradation are still not clear. It was proposed that degradation of these heterocyclic lignin components is initiated by a hydroxylation (Bugg et al. 2011b).

4.4.2 Central Pathways for Lignin Degradation in Bacteria

4.4.2.1 Oxidative Cleavage of Aromatic Rings

The process of aromatic ring cleavage is predominantly aerobic, but anaerobic process exists in nature (Fuchs et al. 2011). Funneling pathways for lignin degradation results into formation of derivatives such as vanillic acid, vanillin, syringate, or guaiacol, and these are further converted into few common central intermediates such as protocatechuate (PCA), catechol, and gallic acid. Protocatechuic acid is the most common intermediate formed during funneling pathways of lignin degradation. This central intermediate acts as substrate for ring-cleaving dioxygenases. The pathway for cleavage of aromatic ring can be divided into *ortho* cleavage and *meta* cleavage on the basis of position of hydroxyl group and their fission in aromatic ring (Bugg et al. 2011b). *Ortho* (intradiol) cleavage takes place between two hydroxyl groups catalyzed by Fe^{3+}-dependent dioxygenase, and *meta* (extradiol) cleavage occurs adjacent to one of the hydroxyl group catalyzed by Fe^{2+}-dependent dioxygenase (Masai et al. 2007; Bugg et al. 2011b). PCA is catalyzed by PCA 2,3-dioxygenase (2,3-PCD), PCA 3,4-dioxygenase (3,4-PCD; intradiol), and PCA 4,5-dioxygenase (4,5-PCD; extradiol). 4,5-PCD pathway has been well studied in *S. paucimobilis* SYK-6. Meta cleavage of catechol gives acetaldehyde and pyruvate while PCA yields pyruvate. Pathways for oxidative aromatic ring cleavage have been shown in Fig. 4.4. 3,4-PCD is most widely distributed among proteobacteria and actinobacteria, but 4,5-PCD is only found in proteobacteria. The β and γ proteobacteria genus *Burkholderia*, *Pseudomonas*, *Xanthomonas*, *Klebsiella*, and *Ralstonia* contains either 3,4-PCD, 4,5-PCD, or both the enzymes (Masai et al. 2007; Bugg et al. 2011b).The pathways for oxidative cleavage of aromatic rings and β-ketoadipate pathway have been shown in Fig. 4.4.

4.4.2.2 β-Ketoadipate Pathway

β-ketoadipate pathway is a highly conserved metabolic route and has been extensively characterized in *Pseudomonas putida*, *Acinetobacter calcoaceticus*, and *Agrobacterium tumefaciens* (Masai et al. 2007). Ortho cleavage of catechol and PCA leads to β-ketoadipate followed by succinate and acetyl-CoA formation (Fuchs et al. 2011; Abdelaziz et al. 2016). β-KA pathway links the lignin-derived upper funneling pathway with TCA cycle through PCA or catechol intermediates (Harwood and Parales 1996; Pérez-Pantoja et al. 2010). There are several enzymes involved in degradation of PCA and catechol to β-KA, and the enzymes are tightly

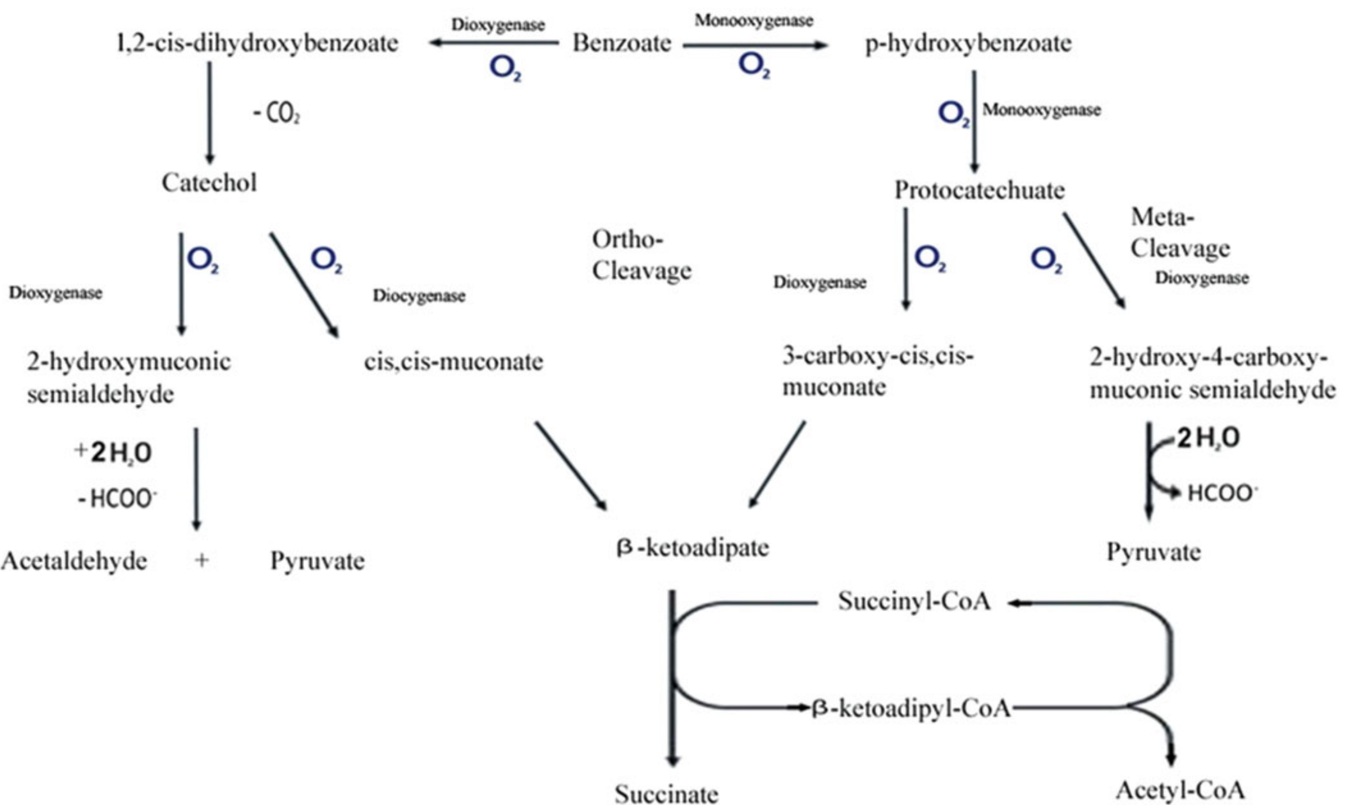

Fig. 4.4 Central aromatic ring cleavage and β-ketoadipate pathway; Dioxygenases cleave the aromatic ring at either ortho (between the two –OH groups) or meta (adjacent to one of the –OH groups) position

regulated (Abdelaziz et al. 2016). Among the several lignin-degrading microbes studied so far, ortho or β-KA cleavage pathway was found to be dominant pathway (Bugg et al. 2011b).

4.5 Bacterial Enzymes Responsible for Funneling Lignin and Its Degradation Intermediates

The major lignin-degrading or modifying enzymes responsible for utilization of lignin or its degradation intermediates are detailed in the following sections.

4.5.1 DyP-Type Peroxidases

DyP-type peroxidases (DyPs) are heme-containing new class of peroxidase recently identified and are predominantly present in bacteria. This enzyme is also present in fungi, first discovered from *Bjerkandera adusta* and named so by studying their activity on anthraquinone and azo-dyes (Sugano et al. 2007). Bacterial DyPs have low redox potential than fungal DyPs, but they showed activity toward phenolic as well as nonphenolic compounds. They can perform catalysis by utilizing H_2O_2 and without H_2O_2 as oxygenase or hydrolase. The first extracellular lignin-degrading peroxidase from bacteria has been reported in *S. viridosporus* T7A, and after genomic analysis it was assumed to be DyP (Ramachandra et al. 1988). Several other peroxidases have been reported from this strain responsible for degradation of *β*-aryl bonds, the most predominant bond in lignin. DyPs were characterized from *Rhodococcus jostii* RHA1, *Pseudomonas fluorescens* Pf-5, *Amycolatopsis* sp. 75iv2ATCC, *Bacillus subtilis* KCTC2023, *Pseudomonas putida* MET94, *Saccharomonospora viridis* DSM 43017, *Thermobifida fusca.* Their broad substrate range (synthetic dyes, Kraft lignin, lignin model compounds, monophenolic compounds, veratryl alcohol, carotenes, Mn^{+2}) and mechanism of action have also been discussed (de Gonzalo et al. 2016;Priyadarshinee et al. 2016; Bugg et al. 2016). The reaction catalyzed by DyPs has been shown in Fig. 4.5.

4.5.2 Laccase (Benzenediol: Oxygen Oxidoreductases; EC 1.10.3.2; AA1)

Laccases are multicopper oxidase that oxidizes various aromatic compounds (phenolics) with reduction of oxygen molecule to water as byproduct. These are extensively distributed among plants, fungi, insects, and bacteria (Riva 2006; Munk et al. 2015). Laccase mostly contains three structural domains, but one or two domains may be lacking in some laccases. Laccase have 4 copper atoms, and these are classified into three groups (type) based on spectral and paramagnetic properties. The type1 (T1) gives blue color (*λ*max. 600 nm), and it is the main site where oxidation of substrate takes place, Type 2 (T2) contains one copper (EPR

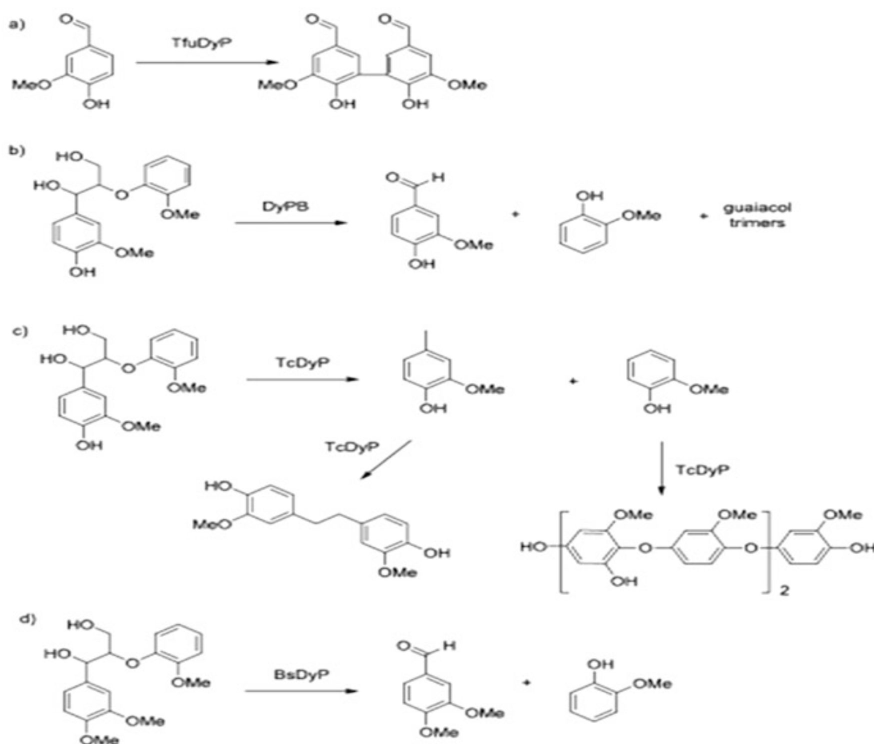

Fig. 4.5 Reaction catalyzed by bacterial DyP-type peroxidases. (**a**) *Tfu*DyP from *Thermobifida fusca* resulted into dimerization of vanillin. (**b**) DyPB from *Rhodococcus jostii* RHA1 formed guaiacol, guaiacol trimmers, and vanillin. (**c**) *Tc*DyP from *Thermomonospora curvata* degraded guaiacylglycerol-β-guaiacol into cresol dimers and hydroxylated guaiacol pentamers. (**d**) *Bs*DyP from *Bacillus subtilis* KCTC 2023 degraded veratrylglycerol-β-guaiacol ether (adapted from de Gonzalo et al. 2016)

active) and Type 3 (T3) contains two copper atoms and together they form trinuclear cluster where oxygen reduction occurs (Bugg et al. 2011b; Munk et al. 2015; Feng et al. 2016). Laccase from fungus has been widely studied and applied but with the advancement in genomics various laccases have been discovered in bacteria (Santhanam et al. 2011; Feng et al. 2016). *Streptomyces* (*S. coelicolor*, *S. violaceusniger*, *S. ipomoea* CECT 3341, *S. ipomoea* CECT 3341, *S. griseus*) has been the most studied bacterial genus for lignin degradation. Various other bacteria such as *Pandoraea* sp. ISTKB, *Bacillus tequilensis* SN4, *Pantoea ananatis* Sd-1, *Bacillus pumilus* (CotA), *Thermus thermophilus* HB27, etc., have also been studied (de Gonzalo et al. 2016; Kumar et al. 2018).

Laccase-mediated oxidation of phenolic β-O-4 (most abundant linkage in lignin) and β-1 has been demonstrated on lignin model dimers. Laccase can perform degradation of phenolic as well as nonphenolic substrate in the presence of mediators. Mediators are small molecules that act as an electron carrier, and upon

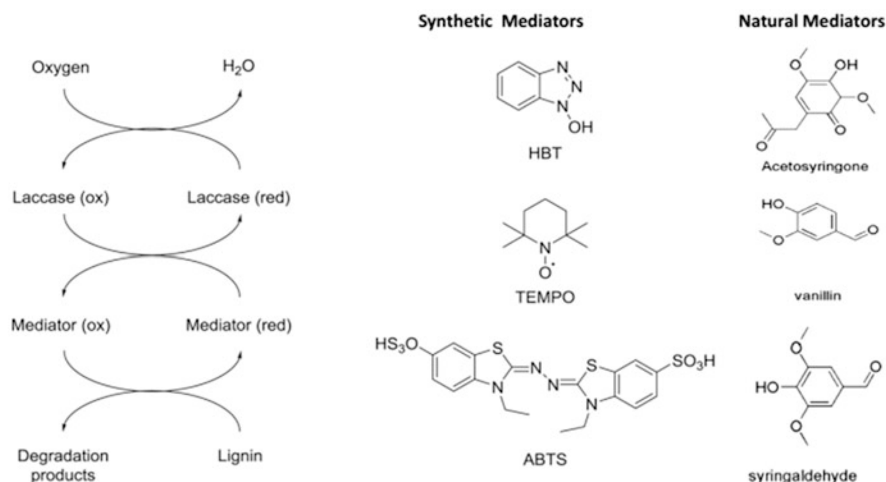

Fig. 4.6 Reaction mechanism of laccase mediator system and some commonly used synthetic and natural mediators. Lignin is degraded by laccase with the help of mediators. Some of the common synthetic mediators are N-hydroxybenzotriazole (HBT), 2,2,6,6- tetramethylpiperidin-1-yl) oxyl (TEMPO), and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and natural mediators are acetosyringone, vanillin, and syringaldehyde

oxidation by laccase they become strong oxidizing intermediates to degrade nonphenolic units and also prevent polymerization of small reactive compounds formed during degradation (Riva 2006; Munk et al. 2015). Mediators may be natural (cinnamic acid, acetosyringone, benzaldehyde, sinapic acid, etc.) or synthetic (ABTS, HBT, TEMPO, etc.). Mechanism of laccase-mediator system and common natural and synthetic mediators are shown in Fig. 4.6.

Since laccases do not require H_2O_2 for substrate oxidation as compared to peroxidases, these enzymes has been used in various industrial applications such as delignification and pretreatment of biomass, wastewater treatment, bioremediation, food and beverages industry, pharmaceuticals and other fine chemicals synthesis, textile dye removal, etc. (Chandra and Chowdhary 2015).

4.5.3 Glutathione-Dependent β-Etherases

The enzyme glutathione-dependent β-etherases were first discovered and studied in detail in α-proteobacterium *Sphingobium* SYK- 6. The etherase enzyme system comprising stereospecific Lig DEF was studied in *Sphingobium* SYK- 6 for degradation of lignin model compounds (Masai et al. 2007). This strain was shown to catalyze the glutathione (thiol group)-mediated cleavage of β-aryl ether linkage in model compounds as shown in Fig. 4.7. The glutathione-dependent cleavage of ether linkages by β-etherase is shown to be enantioselective (de Gonzalo et al. 2016; Feng et al. 2016). Presence of β-etherase in *Novosphingobium*, *Sphingobium* SYK-6,

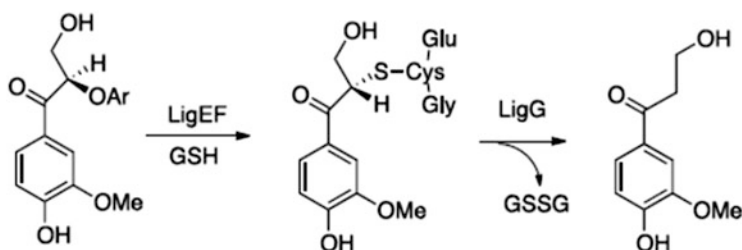

Fig. 4.7 β-etherases catalyzed reaction on lignin model compounds in *Sphingobium* SYK- 6 (adapted from Bugg et al. 2016)

Novosphingobium sp. PP1Y, and *Thiobacillus denitrificans* ATC25259 has been reviewed (de Gonzalo et al. 2016; Feng et al. 2016).

4.5.4 Superoxide Dismutases

Superoxidases are generally intracellular enzymes that protect cells from oxidative damage by converting superoxide anions into molecular oxygen and H_2O_2. Recently two extracellular manganese-dependent superoxide dismutases (MnSOD1 and MnSOD2) have been discovered from *Sphingobacterium* sp. T2 with lignin-degrading properties (Rashid et al. 2015). These two enzymes are shown to be highly active and can oxidize Organosolv, Kraft lignin, and lignin model substrates into various compounds. The degradation products were obtained from oxidative cleavage of aryl-Cα, Cα-Cβ bond, and O-demethylation activity (Rashid et al. 2015).

4.5.5 Catalase-Peroxidases

An extracellular catalase-peroxidase was discovered recently in *Amycolatopsis* sp.75iv2 while growing on lignocellulosic substrate. This is a heme-containing enzyme and showed oxidation of various phenolic model compounds; however, methylated derivatives were not utilized as substrate (Brown et al. 2011). Further research is needed to establish the occurrence of catalase-peroxidases in other strains and their activity to modify polymeric lignin.

4.5.6 Dehydrogenases

The dehydrogenases have been discovered recently in some bacteria capable of transforming lignin or lignin model compound. The enzyme dehydrogenase from strain SG61-1 L has been characterized for degradation of ether linkages in lignin model compound guaiacylglycerol-guaiacyl ether (GGE) and showed very efficient degradation of all its stereoisomers. Enzyme system Lig DEG (LigD-Cα-

dehydrogenase, β-etherase, and glutathione lyase) from *Sphingobium* sp. SYK6 known for the β-O-4-aryl ether linkage has been expressed in *E. coli* and has shown to convert complex lignin structure from hardwood and softwood into monomers (Reiter et al. 2013). Dehydrogenases act on toxic aldehyde (generated during degradation) and convert them into their acids that are less toxic to cells and hence play important part in catabolic funneling (Pérez-Pantoja et al. 2010; Abdelaziz et al. 2016).

4.5.7 O-Demethylases

The enzymes of demethylase system removes methyl group from methoxy-substituted lignin-derived aromatic compounds such as syringate, vanillate, or guaiacol in the presence of cofactors. There are two types of demethylation system. Type one demethylase comprises of an oxygenase (iron-binding site and a Rieske type [2Fe-2S] cluster) and a reductase (a flavin and a [2Fe-2S] redox center) predominant in aerobic microbes such as *Pseudomonas* and *Acinetobacter* (Masai et al. 2007; Bugg et al. 2011b; Abdelaziz et al. 2016). Type two demethylase system catalyzes tetrahydrofolate-dependent demethylation of lignin-derived intermediates mainly reported in anaerobic microbes such as *Acetobacterium dehalogenans* and *Acetobacterium woodii.* After demethylation, the products converge at a few common intermediates (protocatechuic acid, catechol, or gallic acid), which undergo ring cleavage (intra- or extradiol) and are metabolized further by β-ketoadipate pathway (Masai et al. 2007; Bugg et al. 2011b; Abdelaziz et al. 2016).

4.5.8 Dioxygenases

Streptomyces sp. SirexAA-E was found to secrete a fusion enzyme SACTE_2871 containing aromatic ring dioxygenase (intradiol) domain and lignin-binding domain while growing on lignocellulosic biomass. This enzyme contains Fe^{3+} active site and performs oxygen-dependent cleavage of catechol; however, no activity on their methylated derivatives was observed. The secretion is carried out by Tat translocation pathway (Bianchetti et al. 2013). An extradiol dioxygenase was also discovered in the *Sphingomonas paucimobilis* SYK-6 responsible for lignin degradation. This dioxygenase was identified while studying degradation of biphenyl compound as substrate by this strain (Sonoki et al. 2009). These evidences clearly indicate the role of dioxygenase in lignin degradation.

4.5.9 Aromatic Alcohol Oxidase (EC 1.1.3.7)

This enzyme belongs to the class oxidoreductases and attacks on the CH–OH group of the donor, with oxygen as acceptor. It generally attacks the primary aromatic alcohol and converts it into aldehyde. It is a monomeric enzyme mostly found in

fungi such as *Geotrichum candidum*, *Botrytis cinerea, Pleurotus eryngii*, *Pleurotus sajor-caju*, *Pleurotus pulmonarius*, *Penicillium simplicissimum*, *Phanerochaete chrysosporium*, *Brachypsectra fulva*, *Fusarium solani*, *Bjerkandera adusta*, *and Rigidoporus microporus*, and one bacterium *Sphingobacterium* sp. ATM is reported to exhibit this enzyme. Few *Pseudomonas* sp. under anaerobic conditions also show the presence of veratryl alcohol oxidase analog. This enzyme can efficiently modify or degrade aromatic alcohols produced during the degradation of lignin.

4.6 Role of Genomics and Proteomics in Understanding Lignin Degradation

Introduction of new molecular techniques in genomics, transcriptomics, and proteomics and advances in instrumental resolution paved the way for improved understanding of lignocellulosic biomass deconstruction by individual microbes and complex microbial communities. The increasingly available genomic data for bacteria and fungi indicate the potential of microbes for biomass degradation across diverse taxa. Comparative analysis of genome gives information regarding their taxonomic classification and possible physiological prospective (Baldrian and López-Mondéjar 2014). Recent development in NGS applied on lignocellulose-degrading fungi, bacteria, and complex community has been reviewed (Kameshwar and Qin 2016). The genome sequence of individual bacterial strain of actinobacteria, α-proteobacteria, β-proteobacteria, and γ-proteobacteria along with their important genomic features responsible for lignin degradation has been reported (Kameshwar and Qin 2016). Improvement in liquid chromatography and mass spectrometry with quantitative proteomics techniques such as isobaric tags for relative and absolute quantitation (iTRAQ) and label-free quantification (LFQ) has provided a solid platform to quantify proteins and their expression studies.

Enzyme production study at different time points can be performed to study the set of proteins expressed at a specified time under different culture conditions (Baldrian and López-Mondéjar 2014; Singh et al. 2017). Novel ligninolytic enzymes and unannotated proteins responsible for degradation can be identified. In recent studies, NGS is complemented with proteomics and metabolomics to get further precise information regarding pattern of bacterial biomass degradation. The genome sequence of some of the recently reported lignin-degrading bacteria are *Tolumonas lignilytica*, *Pandoraea* sp. ISTKB, *Pseudomonas* sp. strain YS-1p, *Rhizobium* sp. strain YS-1r, and *Burkholderia* sp. strain LIG30 (Woo et al. 2014; Billings et al. 2015; Prabhakaran et al. 2015; Bao et al. 2015; Kumar et al. 2016). Kumar et al. (2018) recently reported genomics and proteomics for understanding the novel genes, differential expression of the important genes on Kraft lignin and vanillic acid (most common intermediate found during lignin degradation). Novel pathways and enzymes were discovered for phenylacetate and benzoate (Kumar et al. 2018). Lin et al. (2016) used proteomics and genomics approach and engineered *Pseudomonas* sp. A514 for efficient lignin utilization and bioconversion. The polyhydroxyalkanoate production efficiency reached 73% of bacterial cell dry

weight in the engineered strain. Several bacteria like *Arthrobacter* sp. Rue61a, *Amycolatopsis* sp. strain ATCC 39116, *Novosphingobium* sp. Strain MBES04, *Cupriavidus basilensis*B-8, *Halomonas* sp. strain KO116, *Klebsiella* sp. strain BRL6-2, *Raoultella ornithinolytica* strain S12 have shown to degrade the lignin and its monomers and are validated by whole genome sequencing and followed by identification of enzymes for lignin degradation and its bioconversion (Kameshwar and Qin 2016; Kumar et al. 2016).

4.7 Conclusion

The addition of value to the lignin generated as waste from plant polysaccharide-based industry will define the success and sustainability of such industries. The biological process is discussed for lignin depolymerization and degradation as it is the most eco-friendly and cost-effective route for its valorization. The pathways for the funneling of heterogeneous lignin derivatives and the enzymes assisting in the lignin utilization were discussed that will further assist in engineering the strains for the enhanced lignin degradation and its valorization. The value addition to lignin will establish the biorefinery concept and also validate the model of circular economy.

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Microbiology of Bioelectrochemical System

5

Surajbhan Sevda, Kaustubha Mohanty, and T. R. Sreekrishnan

Abstract

In the recent time more work is considered in bioelectrochemical system for obtaining power and wastewater treatment. In these systems many microbes are used to carry out the electron transfer from medium to the anode electrodes. These microbes are called electrogen since they have the capacity to transfer these electrons. The main group comes under Geobacter species that provide the high strength to these microbes. The initial source of anaerobic culture is conventional wastewater treatment plants and sludge is collected and used in the anodic chamber of microbial fuel cell (MFC). For improving the performance of MFC, the lag phase of the system needs to be reduced and more current research are going on for understanding of biofilm formation of anode electrode and transfer of their electron to the anode electrode.

5.1 Introduction

In the past decade the bioelectrochemical system (BES) is emerged as a novel technology for wastewater treatment and high value chemical production. There are many groups in this category those are developed for the wastewater treatment and bioelectricity generation. The microbial fuel cell (MFC) is the most studied bioelectrochemical system for wastewater treatment and bioelectricity generation. In these in the starting stage only synthetic wastewater was used and at later stage real

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industrial wastewater is also used for the treatment. The other systems such as microbial electrolysis cell and microbial desalination cells are also developed for the chemical production and seawater desalination, respectively. The microbes play a very important role in these systems for electron transfer and wastewater treatment. These microbes are much specified in the electron transfer from the anodic medium to the anode electrode. This transfer happened either by direct transfer or use of any mediators. The direct electron transfer has several advantages over the mediator electron transfer, as these mediators are costly and also toxic to the environment. Many studies shown that in the direct electron transfer the microbes make a biofilm on the surface of the electrode and then they directly transfer these electrons to the anode electrode. The explanation of the nitty-gritty of microbial extracellular electron transfer (EET) and its utilization in engineered systems is a fundamental and rapidly greater than ever field of research and development (Rabaey and Rozendal 2010; Logan and Rabaey 2012; Sevda et al. 2015). The study of these electroactive (or bioelectrocatalytic or electrochemically active) microorganisms, including pure strains as well as defined co-cultures and complex consortia, requires a complex arsenal of techniques, methods, and protocols (Harnisch and Rabaey 2012; Sevda et al. 2018). These methods derive from diverse methodical disciplines, e.g. materials science, microbiology, electrochemistry, and offer insights on different hierarchical levels, i.e. from the complete microbial biofilm to solitary molecules. Thereby, electrochemistry and electrochemical methods represent the fundament of all behavior. Traditionally, fuel cell type setups were often used for the growth and maintenance of electroactive microbial cultures in the archetype of these engineered systems: microbial fuel cells (MFCs) (Logan et al. 2006; Sevda et al. 2016; Naha et al. 2020). In the traditional types of MFCs often did not allow monitoring or even scheming the potential of a single electrode and thus only limited insights in the electrode processes were possible. Often only open cell potential is reported for the tradition MFC. Now it is more and more approved that the monitoring and control of the potential of single electrode in MFCs represents a clear benefit, not only for fundamental research but also for engineering. Furthermore, with the diversification of the applications of microbial electrochemical technologies that now include, e.g. syntheses, remediation, desalination, and even biocomputing, in the so-called microbial bioelectrochemical systems (BES) (Harnisch and Schröder 2010; TerAvest et al. 2011; Sevda et al. 2018) an external control of individual electrode potentials is often substantial. This control is usually achieved by using external influence sources or potentiostats. The application of potentiostat in BES system provide contol in the operating cell potential and resulting the cathodic reactions can be used for the new product generation from CO_2. This is of high importance as the electrode represents the incurable microbial electron acceptor (for anodes) or electron donor (for cathodes) of the extracellular electron transfer (Lovley 2006, 2011).

Thus the control of the electrode potential enables not only the use of a reproducible microbial culturing conditions but also its tailoring in terms of EET thermodynamics (Schröder 2007; Rosenbaum et al. 2011). This includes the performance parameters maximum current density and coulombic efficiency (CE) as well as EET characteristics. Here the identification of the formal potentials of possible and actual

electron transfer sites will be in focus. The extraction of these parameters is shown on the example of wastewater derived mixed culture biofilms that are dominated by *Geobacter species* (Logan and Rabaey 2012). These wastewater derived biofilms are gained by a simple electrochemical selection procedure as confirmed by copious research groups around the world (Rabaey and Rozendal 2010). The BES connecting microorganisms be a symbol of a major domain of research with applications in waste treatment for energy and chemical generation (Lagarde and Jaffrezic-Renault 2011) or biosensors (Reguera et al. 2005; Sevda et al. 2020). A key challenge to these ends is to make certain the electrochemical communication between viable bacteria and electrodes. For instance, electron transfer has been reported for both monolayer bacteria and conductive biofilms deposited onto electrode surfaces. The biofilm conductivity is attributed either to electrically conductive pili or electron hopping between membrane bound cytochromes or small redox compounds that diffuse between the bacteria and the electrode surface (Reguera et al. 2005). Although natural biofilms offer more than a few advantages in bioelectrochemical applications, "artificial" designs in which the microorganisms are powerless within appropriate matrices are of current interest in order to improve the electron transfer reactions. Moreover, such "artificial" environment could provide a unique opportunity to better control the microorganism strain and environment, with strong interest for both fundamental consideration and applied devices, e.g. whole cell biosensors.

Microbial fuel cells (MFCs) are systems that can convert the chemical energy in organic compounds into electricity using the catalytic action of an anaerobic microorganism. These microorganisms are called exoelectrogens (Dominguez-Benetton 2012) and they produce electrical energy oxidizing organic matter. MFCs generally consist of two parts: an anode and a cathode. Organic compounds are degraded by exoelectrogens on the anode surfaces, and CO_2, electrons, and protons are produced as end products. The generated electrons are delivered to the cathode by bacteria following an external circuit, and protons are also moved through the external circuit for electro-neutrality (Schröder 2007). At the aerobic cathode, water is produced by a reaction among protons, electrons, and oxygen (electron acceptor), and electricity is generated. The microbial fuel cell consists of two compartments, the anodic and cathodic chambers that are separated by a selectively permeable, cation-specific membrane (Fig. 5.1).

The anodic chamber consists of microbes suspended under anaerobic conditions in the analyte and the cathodic chamber contains the electron acceptor. In essence, the electron donor is physically separated from the terminal electron acceptor across the two chambers (Sevda et al. 2018).

Many studies have been conducted on wastewater treatment using MFCs because they can generate electricity and treat wastewater simultaneously (Logan and Rabaey 2012). There are five advantages of MFCs for wastewater treatment. (1) MFCs can convert chemical energy of substrates into electricity directly. (2) A smaller amount of activated sludge is produced from MFCs than from other methods during wastewater treatment. (3) This method is environmentally friendly and

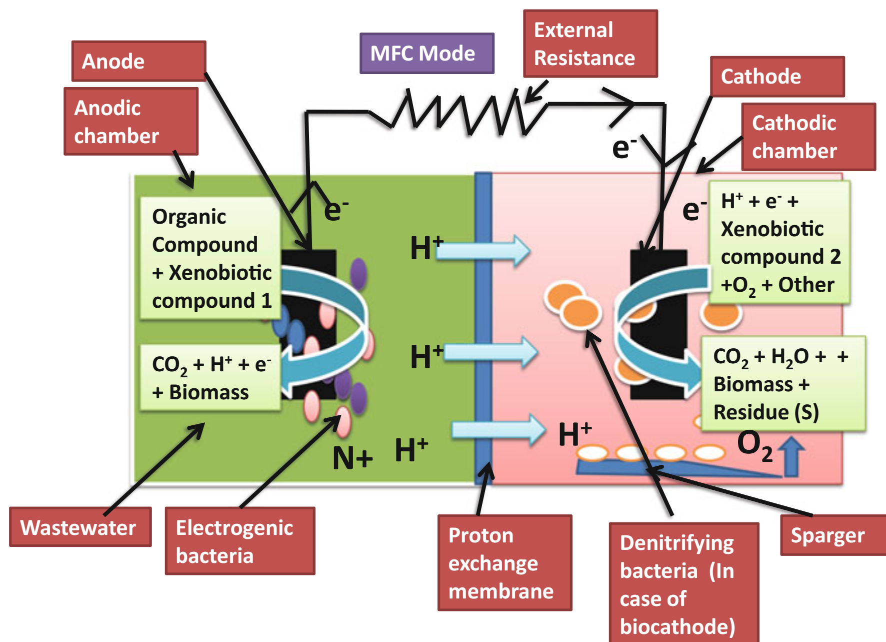

Fig. 5.1 Schematic of microbial fuel cell for bioelectroremediation process for xenobiotic compounds

(4) does not need additional gas treatments. Finally, (5) energy is not necessary for aeration (Logan and Rabaey 2012).

5.2 Microbial Group and Their Quantification for Bioelectrochemical System

Geobacter species play a vital role in the bioelectrochemical system for the electron transfer; the basic mechanism of the electron transfer is direct electron transfer. Bioelectrochemical systems (BESs) are campaigns that harness the electroactivity of microorganisms as power, hydrogen, and/or chemicals, performance guarantee for the processing of and energy recovery from organic wastes. BES performance is at the end of the day dependent on the capability of microorganisms to catalyze redox reactions using electrodes. Not unexpectedly, any parameter that influences the augmentation of BES microorganisms also affects liveliness outputs. The best-studied BESs are those single-minded by anode-reducing bacteria in the genus *Geobacter* (Dominguez-Benetton et al. 2012). The group contains some of the most resourceful exoelectrogens presented in pure culture, and their growth and electroactivity are what in due course drive the catalytic activity of mixed-species anode biofilms in BESs (Schröder 2007). The theory and practice of *Geobacter*-driven BESs, focusing on the model representative *Geobacter sulfurreducens*.

Microbes are an integral part of any bioelectrochemical system (BES). In pure culture systems, they are occupied in catalyzing the transfer of electrons to an anode or from a cathode. In mixed cultures, in addition to these exoelectrogenic and exoelectrotrophic populations, there may be community members involved in other metabolisms that indirectly assist electrochemical activity (e.g., by converting a complex substrate into acetate) or compete with and impair electrochemical reactions (e.g., diverting acetate to methane). A thorough considerate of these systems and the accurate understanding of system performance often require the classification of the microbial communities that institute on either electrode, or perhaps even in postponement or on other reactor surfaces (Schröder 2007). The BES research community has used a multiplicity of techniques to study microbial populations and communities. This chapter focuses on nucleic acid-based methods for community categorization, primarily 16S rRNA and 16S rRNA gene-targeted techniques (although they could be adapted to study other genes), which is consistent with the majority of BES ecology studies. Successful bioelectrochemical systems (BESs) application and research efforts necessitate a comprehensive understanding about the microbial behavior associated with the bioelectrocatalytic conversion of chemical and electrical inputs. BES reactors utilize *living* microorganisms to drive catalytic activity, and these microbes will respond to system changes in poles apart ways than abiotic catalysts. This chapter introduces methods and protocols for quantifying microbial biomass, extracting DNA from electrode surfaces, 16S rRNA gene sequencing, and sequence data analysis. Using these methods, researchers can quantify electron transfer activity per unit biomass and more accurately normalize electricity production results. In addition, 16S rRNA gene sequencing enables researchers to begin taxonomically describing the microbial populations that are present in anode-associated biofilms (Rabaey et al. 2010). The gene sequence data analysis is critical for gaining a basic considerate about the types of microbes that are interacting with electrode surfaces, estimating their function, and quantifying total biomass. These combined data sets can be used as a basis for more detailed analyses relative to microbial function and population dynamics in BESs.

5.3 Functions of Microbial Groups in Bioelectrochemical Systems

The goal of this part is to endow with practical information and fundamental knowledge to researchers who study biofilms on electrodes. The part particularly focuses on cyclic voltammetry (CV) of anodic and cathodic biofilms. We put emphasis on that researchers must obtain cyclic voltammograms during the growth of a biofilm and under no turnover circumstances and put side by side these to known electrochemical controls. We also discussed verifying the suitability of a biofilm reactor setup for electrochemical analysis. We concluded that CV can be more useful in amalgamation with other tools, such as gyratory disk electrodes, quartz crystal microbalance, and microsensors (Dominguez-Benetton et al. 2012). The outcome of any biofilm electrochemical experiment involving a coupled technique relies on the researcher's ability to incorporate the CV analysis discussed in this chapter into a

research plan. Finally, we discuss the implications of microscale gradients in electrochemically active biofilms. In this chapter, a proposed mechanism for long-distance electron transport that occurs over distances that can exceed 20 μm within electrochemically active biofilms comprised of *Geobacter sulfurreducens* wild-type strain DL-1 is described. According to this mechanism, referred to as redox conduction, long-distance electron transport results from sequential short-distance electron transfer reactions ("electron hops") between adjacent redox cofactors distributed throughout the biofilm that act as electron transport conduits. The general approach to investigate the mechanism of electron transport through a material is to place the material between two electrodes and measure the dependency of the rate of electron transport through the material from one electrode to the other, in the form of electrical current, on the potentials applied to the electrodes. Materials utilizing different mechanisms of electron transport exhibit different current–potential dependencies (Dominguez-Benetton et al. 2012). Derivation of the idealized current–potential dependency for a *G. sulfurreducens* biofilm based on redox conduction is described here, which has been successfully applied to fit experimental results. General methodology is also described for performing biofilm electron transport rate measurements in the laboratory. The goal of this chapter is to describe redox conduction and experimental methods to enable researchers to perform electron transport rate measurements for their own types of biofilms. Although biofilms of most microorganisms are electronic insulators, biofilms of pure culture *Geobacter sulfurreducens* as well as mixed species derived from wastewater, when grown on the anodes of microbial fuel cell, are electronically conductive. Remarkably, the electronic conductivity of biofilms is comparable to synthetic conducting polymers (Dominguez-Benetton et al. 2012). Direct conductivity measurements have demonstrated that the biofilm conductivity can be attributed to the network of pili filaments known as microbial nanowires. Surprisingly, the conductivity of pili is metallic-like rather than previously known methods of electron flow in proteins using redox-active cofactors such as c-type cytochromes (Ghach et al. 2014). Electronic conductivity enables microorganisms to access electron acceptors that are many cell lengths away. In addition, biologically produced conductive films, which can be synthesized from inexpensive feedstocks and when alive, can self-repair and replicate, introduce new concepts and materials for bioelectronics. This chapter summarizes the methods used to directly measure the newly discovered conductive properties of biofilms. Firstly, we describe the physical meaning of electronic conductivity (Logan et al. 2006). Then, we discuss three different mechanisms of conductivity in materials—tunneling, hopping, and delocalization. Later, we discuss in detail the experimental methods applied to directly measure conductivity in living biofilms and the results obtained using these methods. We start with four-probe method widely used to measure DC electronic conductivity of materials. Then, we describe a complementary and independent method using two-probe AC impedance spectroscopy to confirm biofilm conductivity and to further distinguish between electronic and ionic conductivity. We summarize the results from different organisms that reveal that biofilm conductivity depends on cell type and physiology. Finally, we discuss two key physical probes—temperature and

gate voltage to probe the mechanism of conductivity in biofilms. The simple method described here to measure electronic conductivity of living biofilms is expected to be a useful tool for future studies of long-range electron conduction in a diversity of microbial systems such that those in sediments and anaerobic digesters.

5.4 Microbial Growth and Death

In microbial electrochemical cells (MXCs), not only classic over potentials known from other types of fuel cells are encountered, but also over potentials associated with the metabolic processes and electron transport pathways in bacteria are encountered. Of the many techniques that can be used to investigate the contributions of various processes to the total over potential in MXCs, this chapter focuses on electrochemical impedance spectroscopy (EIS). This technique is an alternating current technique that involves applying sinusoidal voltage amplitude over a range of frequencies to investigate the processes that control the overall *i–V* response. The use of EIS allows differentiating these various processes, as they are often manifested at different frequencies. We discuss the principles and theory behind EIS, especially with respect to its use in MXCs, as well as important experimental methods and design parameters (Dominguez-Benetton et al. 2012). We show that EIS is an important method that can be used to characterize Ohmic resistance, electrode processes, or an MXC as a whole.

5.5 Conclusion

In the anodic chamber of MFCs, initially, the mixed culture is inoculated from existing anaerobic sludge collected from wastewater treatment. This anaerobic sludge containing anaerobic mixed bacteria and these are responsible for organic removal from the wastewater. In the anodic chamber, they worked in MFC mode and electroactive microbes are accumulated and these electroactive bacteria make biofilm on the anode electrode. So to reduce the lag phase of these microbes, it is recommended that, for a new MFC, starting inoculums should be used from the pre-running MFC. In the biocathodic MFC, denitrifier bacteria are added in the cathodic chamber and anaerobic electroactive bacteria in the anodic chamber of MFC. The electroactive bacteria present in the BES reactor is responsible for its better performance and efficiency for wastewater treatment and bioelectricity generation.

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6 Application of Biotechnology in Oil and Gas Industries

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Abstract

Biotechnology has wide application in medical, agriculture, bioremediation, non-renewable energy production, and food production. Nowadays, environment friendly biotechnological approaches are also popular in petroleum industry. Conventional approaches are high in cost, involve complex implementation process and environmental hazard, while biotechnological approaches are environment friendly. In this chapter, we discuss about biotechnological approaches used by oil and gas industries for resolving production related problems and also for enhancement of production. In oil fields microbial souring, corrosion, and sulfur content in crude oil are major problems faced during or after the production. Another is, production of trapped oil through various enhanced oil recovery processes, in which microbial enhanced oil recovery process has shown great potential. At last, oil sands and shale gas production and its related problems are also been discussed.

6.1 Introduction of Oil Production System

Modernization and technological evolution have developed this era at an exponential phase. With the advent of modern tools and equipment's, the focus on tapping renewable energy potential is the center of every scientific development. However, the non-renewable fossil fuels are still a major requirement of the present world. This global energy demand is presently met by the conventional and non-conventional oil reserves such as onshore, offshore oilfields and oil sands. Currently, the discovery of

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new oil reservoirs along with the development of improved crude oil recovery methods has led to optimal production of crude oil in a sustainable way.

Typically, the recovery of crude oil from these oil reserves proceeds as three different processes: primary, secondary, and tertiary oil recovery. During the primary recovery process, the existence of natural geological pressure is sufficient to produce oil from beneath the earth surface. This pressure leads to the recovery of about 10–20% of original oil in place. However, with time this natural geological pressure gradually decreases and therefore results in reducing percentage of oil been recovered. The secondary oil recovery process is then implemented at this point to re-pressurize the reservoir bottom-hole by water injection. Injection of water in the injection well sweeps the oil along the flow path leading to recovery of crude oil from the production well. About 40–50% of the residual oil in place is recovered during this process (Gieg et al. 2011).

Secondary flooding process is also limited for a period of time. With continuous injection of water, channels are formed along the flow path, termed as *viscous fingering*, which further lowers the efficiency of the recovery process. Moreover, much of the oil still remains in the reservoir, as the water to oil ratio increases in the production fluid and thus leads to unprofitable production of oil. Recovery of the rest percentage of crude oil involves implementation of different methods to recover residual oil based on reservoir nature. These recovery processes are termed as tertiary oil production or enhanced oil recovery (EOR) processes and are typically adopted after secondary flooding. The EOR process is of different types, such as chemical, thermal, miscible gas, and microbial based process. The chemical EOR process (CEOR) involves injection of solvents, surfactant, and mostly polymers such as xanthan gum and partially hydrolyzed polyacrylamide to improve the sweep efficiency of the drive fluid. Thermal recovery process such as SAGD (steam assisted gravity drainage) involves the injection of steam to lower oil viscosity and especially applied during heavy oil recovery. Microbial enhanced oil recovery (MEOR) is further implemented as a tertiary process which is recognized as a greener and sustainable way of oil recovery process. In MEOR, microbial activity is utilized for producing crude oil. Microbes are known to use oil organics for production of biosurfactant, bioemulsifier, biopolymers, various acids and gases. These microbial products aids in the oil recovery process by improving the efficiency of the drive fluid (Gbadamosi et al. 2019).

Additionally, two different types of oil fields are present throughout the world, i.e., onshore and offshore oil fields. Onshore oil fields are those fields discovered on the land, while offshore oil fields are found in the sea. Both these oil fields constitute similar oil production systems that are composed of two types of facilities. The downhole facilities include injection and production well components, while above ground facilities comprise injection pipelines, production pipelines, and processing terminal facilities. Water during the secondary oil recovery process passes from the processing terminal to the injection well, and then is injected downhole which leads to its recovery from the production well. The produced water then passes through the three-phase separator and finally to the processing terminal where oil is separated. This whole secondary process, therefore, requires large amounts of injection water.

This large quantity is generally drawn from nearby water aquifers (e.g., sea, river, and lake or ponds) and sewage treatment plants. Besides, the onshore oil fields adopt a sustainable process, produced water re-injection (PWRI), wherein the produced water after oil separation is reused for re-injection in oil field. The offshore oil fields, on the other hand, have access to large sums of sea water, and, therefore, do not require PWRI strategies (Prajapat et al. 2019).

6.2 Microbiology of Oil and Gas Industry

Microbes are ubiquitous in nature and are present in a variety of extreme environments. However, earlier beliefs added oil and gas industries as an exception, till the nineteenth century, when the microbiology of oil reservoirs was introduced (Bastin et al. 1926). It was found that the oil reservoir heterogeneous nature itself provides different extreme environments such as high pressure, temperature, salinity, and depth barriers for the growth of organisms. There are varied microbial communities thriving in different oil reservoir zones. Some of these communities are sulfate reducing bacteria (SRB), fermentative bacteria, nitrate reducing bacteria (NRB), iron reducing bacteria (IRB), iron oxidizing bacteria (IOB), and methanogens. These bacterial groups are known to perform their metabolism based on different hydrocarbon sources. Hydrocarbons or oil organics present in the reservoir act as energy sources for these microorganisms with inorganic molecules as an electron acceptor.

Among the different microbial communities, SRB are known to be the dominant species present in the oil fields (Voordouw et al. 1996). Many studies have also stated, they are indigenous to the oil reservoir (Stetter et al. 1987), however, controversies exist whether SRB are indigenous to oil fields or are been introduced with injection water. Though, the existence of similarities between the oil field and hydrothermal SRB population shows the mobility/transportation of SRB communities between two geological distinct environments (Stetter et al. 1987; Voordouw et al. 1996). SRB communities are further known to belong to both eubacterial and archeal lineages. Besides SRB population two different types of NRB are present in the oil reservoir, i.e., heterotrophic nitrate reducing bacteria (hNRB) and sulfide oxidizing nitrate reducing bacteria (so-NRB). The hNRB are known to perform dissimilatory heterotrophic nitrate reduction (into N_2 or ammonia) with the oxidation of oil organics, while so-NRB reduce nitrate into N_2 or ammonia with the oxidation of sulfide into elemental sulfur or sulfate. The NRB abundance is privileged when the nitrate is injected in the reservoir in order to control microbial souring.

The iron reducing bacteria (IRB) and oxidizing bacteria (IOB) are yet other dominant bacterial groups present in various oil fields setup. Iron reducing bacteria uses ferric iron as a terminal electron acceptor, while iron oxidizing bacteria are found to oxidize the ferrous ions. Additionally, IOB are known to oxidize the Fe under different set of conditions, e.g., acidic, neutral pH, phototrophically, anaerobically, autotrophically, and heterotrophically (Ionescu et al. 2015).

The methanogens are strict anaerobic archaea that produce methane during anaerobic respiration. Methanogens are found in deeper regions of oil reservoirs, utilizing the acetate, one carbon compounds, and H_2 and CO_2 (Prajapat et al. 2019). Generally, methanogens are present with syntrophs (an obligate proton producing bacteria).

6.2.1 Sulfate Reducing Bacteria

Sulfate reducing bacteria (SRB) are morphologically diverse and anaerobic organisms obtaining energy by dissimilatory reduction of sulfate to sulfide (Bernardez and De Andrade Lima 2015). The SRB and sulfate reducing archaea (SRA) are together known as sulfate reducing prokaryotes (SRP). During the sulfate reduction process, these SRP communities oxidize the organic compounds (oil components in the oil reservoir) and reduce sulfate into hydrogen sulfide (H_2S). SRP are also found to have the ability to utilize other sulfur compounds such as sulfite, dithionite, thiosulfate, trithionate, elemental sulfur, and polysulfides.

The sulfate reduction processes are of two types, assimilatory sulfate reduction and dissimilatory sulfate reduction. During assimilatory sulfate reduction, the final product of sulfate reduction is used for the biosynthesis of sulfur containing biomolecules, while dissimilatory sulfate reduction produces H_2S which is released in the surrounding environments. Dissimilatory sulfate reduction is a three step process: In the initial step, sulfate is reduced into adenosine-5′-phosphosulfate (APS) with the help of the ATP sulfurylase enzyme. Then, APS is converted into sulfite and adenosine monophosphate (AMP) with the help of APS reductase enzyme. During the last step, produced sulfite is further converted into H_2S with the help of dissimilatory bisulfite reductase (Dsr) enzyme (Prajapat et al. 2019).

SRP communities are known to be phylogenetically distributed variably along the oil fields. Such as, the presence of SRB depends upon the oil field origin, oil composition, downhole temperature, pH of oil–water mixture, depth of the reservoir, stage of oil production, and salinity of produced water. The variance of SRP communities also differs worldwide in different oil fields. The general SRB communities present in varies oil field setup are *Desulfotomaculum, Desulfosporosinus, Thermodesulfobacterium, Thermodesulfovibrio, Thermodesulfobium, Thermotoga, Thermodesulfohabdus, Desulfacinium, Desulfobulbus, Desulfocapsa, Desulfofustis, Desulforhapalus, Desulfobotulus, Desulfotignum, Desulfobacter, Desulfobulbus*, and *Desulfovibrio* (Prajapat et al. 2019). While SRA present in various oil fields are *Archaeoglobus fulgidus, Archaeoglobus profundus, Archaeoglobus veneficus,* and *Caldivirga maquilingensis* (Prajapat et al. 2019).

Moreover, beside there divergence the SRP population are widely distributed among the oil field facilities. These microbes thrive in-between the oil and water phases. They mostly inhabit in the injection, production, and crude oil processing facilities. As these SRP population grow in different environments, they showed varied types of physiology. The SRP present in the oilfields are mesophilic,

thermophilic, halotolerant, and halophilic in nature. They are the causes for corrosion of pumping facilities and storage tanks, lower quality of oil products, e.g., sulfur content and a number of limiting factors.

6.2.2 Iron Reducing and Oxidizing Bacteria

Iron reducing bacteria (IRB) reduce the ferric iron (Fe^{3+}) into ferrous (Fe^{2+}) iron with the oxidation of organic substrates. This reduction of ferric can be coupled with the fermentation process. Besides, the ferric iron acts as a terminal electron acceptor, and with other terminal electrons can be used by the IRB (e.g., sulfate). Moreover, as iron is insoluble in nature; therefore, during the iron reduction process, membrane bound ferric reductase enzyme is present in the IRB (Lovley 1993). Additionally, the assimilation of iron after reduction is not possible in IRB. Also, the phylogeny of IRB is closely related to SRB phylogeny as some IRB have the capabilities to reduce sulfur. However, in the presence of H_2, IRB outcompete SRB and methanogens (Fredrickson and Gorby 1996). Examples of IRB are *Geobacter, Shewanella, Desulfuromonas,* and *Pelobacter*.

Iron oxidizing bacteria (IOB), on the other hand, oxidize the ferrous iron (Fe^{2+}) into ferric iron. These types of bacteria are generally microaerophilic in nature. They are also found to be autotrophic, heterotrophic, and mixotrophic in nature (Bridge and Johnson 1998). Examples of some IOB species are *Sulfobacillus thermosulfidooxidans, Sulfobacillus acidophilus, Acidimicrobium, Gallionella ferruginea, Leptothrix, Sideroxydans, Mariprofundus ferrooxydans,* and *Ferritrophicum radicicola*. One hypothesis also concludes that IOB indirectly involve microbial influenced corrosion by deoxygenation and lead to SRB growth. Thus, these communities are actively involved in corrosion (Emerson et al. 2010).

6.2.3 Nitrate Reducing Bacteria

Nitrate reducing bacteria (NRB) use nitrate as a terminal electron acceptor and contribute in the nitrogen cycle. Generally, NRB are heterotrophic in nature and can be facultative anaerobes in nature, sometimes. Also, few of the NRB species can grow autotrophically with the oxidation of sulfur and iron containing compounds, and use carbon dioxide or bicarbonate as the carbon source (Matějů et al. 1992). These different types of their metabolism switch according to the surrounding environmental conditions. NRB respire nitrate in the limiting oxygen condition through the membrane bound nitrate reductase enzyme. Fewer NRB species can also oxidize the sulfide into sulfate or sulfur elements, and are known as sulfide oxidizing nitrate reducing bacteria (so-NRB). In the oil fields, NRB activity is induced when the nitrate is injected as a strategy for controlling microbial souring. During injection of nitrate, NRB outcompete the sulfate reducing bacteria for similar energy source (oil components). Besides, the so-NRB activity also increases during this process and decreases sulfide concentration in the production fluids by sulfide

oxidation. Some of the NRBs are also capable of incomplete reduction of nitrate under stress conditions such as temperature, high nitrate concentration in environment which leads to conversion of nitrate to nitrite only. The nitrite so produced is a strong inhibitor of the Dsr enzyme of SRB and therefore limits the growth of SRB population. Besides, nitrate reduction occurs at a high redox potential which also limits SRB growth with increasing redox potential.

6.2.4 Methanogens

Methanogens are strictly anaerobic archaea which produce methane as a by-product during metabolism. These organisms can be present in any part of the sediments when the sulfate is depleted. Methanogens typically use methanol, methylamines, methyl sulfate, formate, $H_2 + CO_2$ or CO and acetate as a carbon and energy source (Schlegel and Müller 2011). They are of two types: acetoclastic methanogen and hydrogenotrophic methanogen. Acetoclastic methanogens use acetate, while hydrogenotrophs use hydrogen and CO_2. These communities are present in swamps, digestive systems of animals, marine sediments, hot springs, hydrothermal vents, and oil fields.

Methanogens belong to the phylum Euryarchaeota in the archaea domain. They are further classified into seven orders: Methanococcales, Methanobacteriales, Methanosarcinales, Methanomicrobiales, Methanopyrales, Methanocellales, and Methanomassiliicoccales (Enzmann et al. 2018). In Methanosarcinales order acetoclastic methanogens are present, while in order Methanococcales, Methanobacteriales, Methanomicrobiales, and Methanopyrales hydrogenotrophic methanogens are present. Methylotrophic methanogens use methylated compounds like methanol, methylamines, or methylated thiols. These are present in the order Methanomassiliicoccales, Methanobacteriales, and Methanosarcinales.

6.2.5 Acid Producing and Fermenting Bacteria

Acid producing bacteria (APB) produce the acids (e.g., sulfuric acids) during their metabolic activities. This produced acid causes corrosion in the oil field installations. These are fermentative bacteria known to gain energy from the substrate level phosphorylation. These bacterial population use a wide range of organic compounds like sugars, peptides, amino acids, and organic acids. Many reports have also shown APB to use inorganic sulfur compound, ferric ions, and nitrate as a terminal electron acceptor. Besides, fermentative bacteria can grow at wide ranges of salinity and temperature conditions. Mesophilic, saccharolytic fermentative *Halanaerobium* uses disaccharide and monosaccharide in fermentative pathways (Ollivier and Magot 2005). Fermentative bacteria in oil fields are present in separators and filters where extensive involves in biodegradation of oil. Thermophilic fermentative bacteria are also present in oil fields and belong to the order Thermotogales (Prajapat et al. 2019).

6.3 Oil Field Souring

Biogenesis of hydrogen sulfide (H_2S) by SRB (known as a souring) has a deleterious effect on the human which could lead to death after inhalation. This anthropogenic H_2S generation is not only dominated in the oil industry but also found in paper and pulp industry, rayon textile production, chemical manufacture, and waste disposal. Annual cost estimated $90 billion associated with the microbially produced H_2S in reservoir and fluids (Gieg et al. 2011). While, injection of water during the secondary recovery is found to be mostly responsible for souring in oil fields. The probable reason for such an effect could be the sulfate content in the injection water used by oil industries. Water sources used for secondary recovery are seawater, municipal waste water, and the saline aquifer that contain moderate to high sulfate concentration (5–30 mM). In offshore oilfields the seawater are injected which contain high sulfate concentration of 25–30 mM (Voordouw and Grigoryan 2009). Sulfate containing water injected in an oil reservoir is conducive for SRB activity in the reservoir matrix and leads to conversion of sulfate to sulfide. While, injection water also act as a source of both inoculum and electron acceptor. Therefore, both indigenous and adventitious populations thrive in reservoir rock and are responsible for reservoir souring. Also, during the secondary oil recovery process, water-flooding decreases the temperature of the reservoir and also dilutes the harmful petroleum fluids. These further creates a zone at the near injection wellbore region (NIWR), making it a suitable condition for boosting microbial growth.

Additionally, in the reservoir, more labile electron donors are present in the form of simple organic acids (acetate, propionate, butyrate, etc.) at high concentrations as much as 1500 mgL^{-1} (Vance and Thrasher 2005). Besides utilizing these electron donors, SRB are also capable to utilize diverse aliphatic and aromatic hydrocarbon (Anderson and Lovley 2000; Annweiler et al. 2000; Abu Laban et al. 2009). Therefore reservoir conditions provide a limitless carbon and energy supply to activate microbial population. The composition or quality of injection water is a key factor to the extent of SRB activity in situ. Produced water re-injection (PWRI) strategy also provides the soluble oil organics like volatile fatty acids (VFA). VFAs include acetate, butyrate, and propionate and other organic, for example, lactate. VFAs are completely oxidized by SRB in CO_2 and incompletely oxidized into acetate that is excreted into the environment (Widdel and Rabus 2001).

The large quantities of H_2S generated by SRB cause a variety of problems (Larsen 2002), such as contamination of crude oil, metal corrosion, and the precipitation of metal sulfides. The souring in production facilities and the reservoir leads to additional costs associated with the prevention of operators exposure to toxic H_2S, reduced oil–water separator performance, management of iron sulfide solids, and accumulation of iron sulfide deposits that are responsible for the enhancement of equipment corrosion and fouling of equipment corrosion (Vance and Thrasher 2005). Sulfide production has been broadly classified to have three negative impacts; firstly, the toxicity of sulfide as a concern to the worker and public health. Secondly, sulfide production decreases the oil quality, and, therefore, sulfur content must be lowered by processing facilities. Lastly,

sulfide is corrosive in nature, which causes leaks or ruptures in pipelines; increases the potential of environment damage in a remote location and negatively impacts the facility integrity. Also, the sulfide production can arise abiotically by thermo-geochemical processes, but water injection induces the souring significantly by increased SRB activity. The distinguishing character between this biological and non-biological sulfide production can be identified by isotope signature analysis of the ratio of ^{34}S to ^{32}S (Frazer and Bolling 1991).

6.3.1 Souring Control by Traditional Methods

Prevention of souring can be done by choosing suitable make-up water sources without sulfate, but this is generally not possible as oil companies have to depend on the nearby water source. Additionally, as a prevention measure the sulfate contained in the injection water can be physically removed by nanomembrane filtration or by reverse osmosis (Robinson et al. 2010). This protective control technology has earlier been adopted by some of the North Sea oilfield platforms (McElhiney and Davis 2002; Odgen et al. 2008). However, the drawback of this technology is the high cost of installation and maintenance of the filtration system. Therefore, commonly used methods are the application of a chemical to terminate sulfate reduction or removal of sulfide from injection water. Chemical sulfide scavenger involves triazines, sodium hydroxide, aldehyde, metal oxides, and nitrite (Vance and Thrasher 2005). These chemical treatment removes sulfide from the downstream operation, but are unable to shortfalls of inhibiting sulfide production at the NIWR. The biocide injection process is regularly followed in the oil industry. Regular treatment of injection and produced water inhibits a broad spectrum of the attack on the particular microbial community. However, repeated dosing of biocide develops the biocide resistance community and therefore alternate injection strategies are proposed (Telang et al. 1998).

Depending on their function these industrial biocides are broadly divided into two categories: oxidizing and non-oxidizing biocide. Oxidizing biocides are ozone and chlorine; these biocides are reactive with microbes, organic matter, solids, and pipeline material. Non-oxidizing biocides are tetrakis hydroxymethyl phosphonium sulfate (THPS), glutaraldehyde (Glut), acrolein, bronopol, and quaternary ammonium salts (Fig. 6.1).

Glut is the second most used biocide in oilfields after the THPS. The use of Glut has found to develop resistance in microbes; therefore, alternation of dosage of biocide has been used to prevent such occurrence (Gieg et al. 2011). Two reactive terminal aldehyde group of Glut cross link amino and sulfhydryl group of proteins and nucleic acids. Quaternary ammonium compounds such as benzalkonium chloride (BAC) work as surfactant, reacting with the cell membrane and rupturing it. THPS is a biodegradable and less toxic compound (Downward et al. 1997) and also dissolves ferrous sulfide precipitation. The mechanism of THPS action is still unknown. Bronopol is an alcoholic compound and inactivates the sulfhydryl group containing protein via the free radical of electron deficient bromine atom (Legin

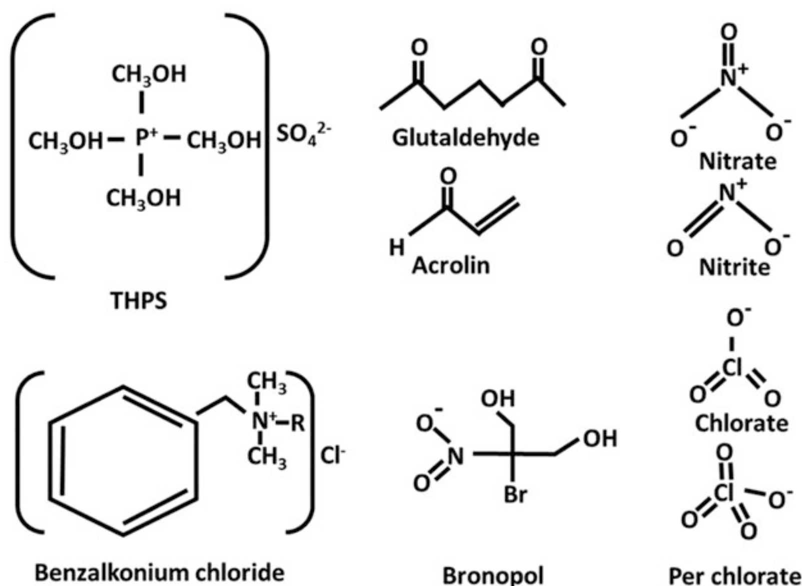

Fig. 6.1 Structure of different types of biocides used in the oil industry

1996). Bronopol is soluble in water but not in a hydrocarbon solvent. This is also tested in laboratory and field study of the oil industry (Tischler et al. 2010). Acrolein is a three carbon unsaturated aldehyde and high chemical reactivity. It has biocidal activity with the activity of scavenging sulfide and also dissolves iron sulfide precipitation. All the biocides have been well studied and different injection schemes have been studied by various researchers to predict the efficiency of various biocides in controlling sulfide production (Penkala et al. 2006).

6.3.2 Souring Control by Nitrate Treatment

From the past many years, nitrate injection is used as a "green" technology to control microbial souring in sewage treatment facilities. Though, the first oil field trials of nitrate injection occurred in the 1990s (Jenneman et al. 1999). Successful application was later reported for both offshore and onshore oil operations (Dinning et al. 2005; Sturman et al. 1999; Thorstenson et al. 2002; Larsen et al. 2004; Kuijvenhoven et al. 2007; Bødtker et al. 2008; Voordouw and Grigoryan 2009). Few of the benefits of using nitrate are, it is cheaper and does not harm the workers and environment. Also, nitrate effectively penetrates the SRB biofilms (Gardner and Stewart 2002) and controls biosouring by different mechanisms. High redox potential of nitrate than sulfate, favors bio-competitive exclusive growth of heterotrophic NRB (hNRB) that outcompetes SRB for the same electron donors (VFA or oil organics) (Hubert et al. 2005; Sunde and Torsvik 2005). Alternatively, nitrate allows the growth of sulfide

oxidizing nitrate reducing bacteria (so-NRB) which oxidize sulfide into sulfate coupled with nitrate reduction. Hence, the sulfate reduction into sulfide by SRB and re-oxidation into sulfate by so-NRB are complementary metabolic cycles.

The reduction of nitrate by hNRB and so-NRB forms metabolic intermediate, i.e., nitrite which further inhibits the SRB growth. Since the structure of nitrite acts as a sulfide analog, it therefore easily binds with dissimilatory sulfite reductase (Dsr) enzyme, which is responsible for sulfate reduction in SRB. This interaction further inhibits the Dsr enzyme thereby limiting the sulfide production. Also, excess accumulation of nitrite can chemically oxidize the sulfide into sulfur, polysulfide, and sulfate (Sanders and Sturman 2005; Kaster et al. 2007; Lin et al. 2009). In such reaction, nitrite is also reduced in N_2 and NH_4^+. Besides, an increase in redox potential by conversion of nitrite to NO and N_2O also inhibits the SRB growth (from −400 mV to +100 mV) (Nemati et al. 2001; Hubert et al. 2009). Moreover, limiting nitrate concentrations during nitrite reduction continuously leads to end products N_2 and NH_4^+ formation (Hubert and Judd 2010). NRBs are known to catalyze the reduction of nitrate via two main pathways in the presence of carbon and energy sources. Conversion of ammonium by dissimilatory nitrate reduction or de-nitrification of nitrogen results in the reduction of nitrate. In the oil reservoir, both these processes of nitrate reduction have been observed (Gevertz et al. 2000; Hubert and Voordouw 2007).

The use of nitrate for controlling souring has different advantages. In general, the biocide treatment has its efficacy against all the resident microbial population present in the reservoir. However, nitrate is known to target SRB specifically and thus can have an added advantage over biocides. Though nitrate can also have some negative effects such as accumulation of biomass leads to pipeline bio-fouling and downstream corrosion due to the change in oxidizing potential (Martin 2008). Dissimilatory nitrate reduction present in the facultative organism also utilizes nitrate as an alternative to aerobic or fermentative growth. Some of the SRB are known to upregulate the nitrite reductase (nrf) gene which detoxifies nitrite to ammonium whereby down-regulating Dsr gene (Haveman et al. 2004). This strategy is not much common among SRB, though nrf transiently inhibits the sulfate reduction by nitrite. Some SRB, for example, *Desulfovibrio gracilis* has the ability to reduce nitrate in the presence of high sulfate concentration.

In comparison to nitrate, nitrite acts as a good inhibitor because it directly inhibits the Dsr enzyme. Some successful field trials have also demonstrated the nitrite application as a biocide for the inhibition of microbial souring, e.g., New Mexico field (Sturman et al. 1999). However, nitrate is easier to handle and is less toxic than nitrite. Additional researches have also proposed the synergistic effect of biocide with nitrate injection. Nitrite and other biocide synergy have also been tested with *Desulfovibrio* sp. SRB consortium. Few reports have mentioned, injection of biocide with a nitrite combination or mixture of different biocides can inhibit the SRB community successfully (Greene et al. 2006).

6.3.3 Souring Control by Novel Biotechnological Approaches

By Injection of Bacteriophage for Killing the SRB Bacteriophage is a virus that infects the bacteria specifically. In order to control souring, these viruses can act as a good approach for bacteriocidal activity. Summer et al. (2017) patented method for prevention and remediation of reservoir souring and corrosion by treatment of virulent bacteriophage (Summer et al. 2017). Their approach was to control souring by specifically targeting the problematic bacteria by virulent lysogenic bacteriophages. These problematic microbes are sulfate reducing bacteria and acid producing bacteria that cause souring and corrosion in oil fields.

By Injection of (per)Chlorate Perchlorate and chlorate (combinedly known as a (per)chlorate) are new metabolic inhibitors that can be applied in the oil fields for souring control. (per)chlorate oxyanion is present in nature and anthropogenically synthesized due to its wide uses (Liebensteiner et al. 2014). Perchlorate (ClO_4^-) is a soluble anion that has a central chlorine atom and is surrounded by four oxygen atoms. To control souring (per)chlorate has the following effects: (1) Bio-competitive exclusion (2) Oxidation of sulfide, (3) Inhibition of sulfate reduction pathway (Okpala and Voordouw 2018).

6.4 Microbial Corrosion Control

Besides souring, corrosion is one of the major problems faced by the oil industries. Corrosion can be CO_2 mediated corrosion, oxygen corrosion, galvanic corrosion, crevice corrosion, erosion corrosion, and microbiological induced corrosion (MIC) (Popoola et al. 2013). Among all the different types of corrosion, microbiologically influenced corrosion occurs mostly in the oil fields. Microbiologically influenced corrosion occurs due to microbial activities. Microbial activities produce CO_2, H_2S, and organic acids and corrode the pipelines. The general principle for the working of corrosion inhibitors to inhibit corrosion is the formation of protective barriers to the metal/metal oxide through chemisorption, physisorption, complexation, or precipitation. Corrosion inhibitors either neutralize the action of corrosive substances or form protective films/stabilizing the pre-existing protective films. Therefore, they prevent the access of oxygen to the cathode, prevent the hydrogen diffusion from the cathode, or inhibit the dissolution of the metal. Inhibitory efficiency of corrosion inhibitors depends on the pH, temperature, duration, metal composition of pipeline, and the structural properties of inhibitor molecules (Lukovits et al. 2001). Corrosion inhibitors also slowdown the corrosion reaction. In the biological context, some corrosion inhibitors prevent the adhesion of microbes to metal surfaces (Videla and Herrera 2009).

6.5 Microbial Enhanced Oil Recovery (mEOR)

Microbial enhanced oil recovery is the biotechnological approach for the tertiary oil recovery process. This is applied by oil industries after the secondary oil recovery process or some times during secondary oil recovery. mEOR process involves use of microbes for enhancing residual oil production from the reservoir. In mEOR, microbes enhance oil recovery by producing gas (CO_2, CH_4, H_2, and N_2), low molecular weight acids, solvent, biosurfactant, polymers, and biomass production (Kaster et al. 2012). These microbial products contribute to the enhanced oil recovery process by different mechanisms. During the mEOR process, either microbial product (mEOR chemicals) may be injected in well or nutrients are added in the injection water for in situ microbial growth. Adding microbes or microbial products in an oil well is also known as bioaugmentation, while adding growth supplement for increasing in situ microbial growth is termed as biostimulation.

During the mEOR process, biosurfactants mobilize the oil by reducing the interfacial tension (IFT) between oil and water phases. Another mechanism is microbial growth on reservoir rocks and biofilm formation changes its rocks wettability. Further microbial growth in reservoir rocks leads to bioclogging of the water flow path. This bioclogging alters the water flow path and ultimately increases a reservoir sweep efficiency. Other than these mechanisms, microbes generate gases during metabolism (for example, methane and carbon dioxide) and these gases increase pore pressure. These gases also dissolve in the oil phase, therefore reduces the viscosity and swelling of the oil. Another mechanism is the biodegradation of the hydrocarbon. Microorganisms utilize the crude oil components as a carbon source and alter oil viscosity (Armstrong and Wildenschild 2012).

6.5.1 Conventional Methods of EOR

Conventional EOR methods are: thermal (steam injection, in situ combustion), chemical (surfactant, polymer, alkali and solvents injection), and miscible gas (CO_2) injection.

Thermal EOR (TEOR): This is a commercially available technique of EOR. In this process, steam is generated from water using natural gas. This steam is injected in the reservoir to heat heavy oil, for reducing its viscosity and also impact on thermal expansion and crude oil vaporization. This process allows the oil to flow easily. Other than using natural gas, alternatively solar energy is also used for steam generation. This TEOR process is unsuitable where thin pay zone and increased depth are present (Gbadamosi et al. 2019).

Chemical EOR (CEOR): CEOR enhances oil production by altering oil–water/oil–rock interactions in the reservoir. Chemical is injected in water slug that lowers the IFT between fluids (water–oil), alter the wettability of rock to increase oil permeability, and increment in the viscosity of injection fluid improves the mobility.

Traditional CEOR methods are polymer, surfactant, and alkaline flooding. In polymer flooding, polymer increases the viscosity of injected fluids and further

increases mobility. In surfactant flooding, surfactant reduces the IFT of injected fluid, thus mobilization of residual oil improves oil production. In alkaline flooding, the pH of injected fluids is increased by the addition of alkaline agents (sodium carbonate, sodium silicate, sodium hydroxide, and potassium hydroxide) (Kumar et al. 1989). Other than these, a binary mix of alkaline-surfactant (AS), surfactant/ polymer (SP), alkaline/polymer (AP), and alkaline/surfactant/polymer (ASP) methods are also applied in tertiary chemical enhanced oil recovery. Combined CEOR methods have improved efficiency for more oil production.

Other than traditional CEOR nanofluids flooding in the field has also been evaluated by few researchers (Gbadamosi et al. 2019). Injected nanofluid contributes to wettability alteration, IFT reduction, and improved viscosity of the injected fluid. Nanofluid flooding can be combined with polymer flooding. Polymeric nanofluids flooding improved rheological properties, increases stability at high temperature and salinity conditions.

Miscible CO_2 gas injection is yet another EOR method in the tertiary oil recovery process. CO_2 has unique displacing properties; it is due to low minimum miscibility pressure with various types of crude oil. During CO_2 injection, it forms a single-phase fluid with hydrocarbon and disrupts the capillary effect to retaining oil in place. CO_2 injection also promotes oil swelling, reduces fluid viscosity, and increases oil mobility.

6.5.2 Novel Approaches for mEOR Using Toluene and Nitrate

During mEOR process, molasses and nitrate are also injected in the reservoir to stimulate the growth of residents and introduced bacteria. Other than these, toluene addition (light oil component) can be a good option, where toluene is present in limiting concentration. Toluene is the preferable energy source of nitrate reducing bacteria. Molasses is used in mEOR due to its high solubility in water and insolubility in oil, while toluene is oil soluble and low molecular weight mEOR substrate. Hence, toluene binding to oil is prevented from been lost during water injection. Gassara et al. (2015) investigated the effect of toluene and nitrate injection on mEOR in laboratory scale (Gassara et al. 2015). They found that additional oil can be produced from heavy oil containing bioreactors. In these bioreactors, they stimulated the hNRB activity by the injection of aqueous nitrate and toluene. The mechanism behind this lab trial is the biosurfactant and biopolymer production by microbes and the stimulation of microbial activity by sequential addition of toluene and nitrate.

6.5.3 Polymers for Enhanced Oil Recovery

Polymer injected during polymer flooding can be categorized into two categories: synthetic polymers and biopolymers. Synthetic polymers are polyacrylamide or its derivatives (hydrolyzed polyacrylamide) and other copolymers of acrylamide. There are a number of biopolymers tested for EOR efficiency such as xanthan gum,

scleroglucan, welan gum, schizophyllan, mushroom polysaccharide, cellulose, and lignin. Biopolymers have added advantage over the synthetic polymer like biopolymer has more rigidity, increased viscosity at higher temperature, superior tolerance to salts/temperature, outstanding thickening capability and stability in harsh condition of reservoirs. Along with these mentioned benefits biopolymer also has limitations like well plugging due to cell debris, bacterial sensitivity, and poor filterability. Other than these, nano-celluloses based nanofluids have better capability to involve in oil displacement (Pu et al. 2018). During the selection of polymer/ biopolymer different rheological properties to be tested at each field condition.

Xanthan is a bacterial polysaccharide and has rigid polysaccharide chains, thus less sensitive to mechanical shear, elevated salinity, and divalent ion concentration. Demerits of using xanthan gum are its high susceptibility to biodegradation and high risk of plugging in rock pores (Pu et al. 2018; Gbadamosi et al. 2019). Other biopolymers such as scleroglucan, produced by fermentation of a plant pathogen fungus (genus Sclerotium) are also been proposed as EOR agent. This is a good substitution of HPAM due to its good solubility and eco-friendly nature. This biopolymer is durable to various pH and high mineralization. Scleroglucan has an excellent viscosifying ability due to its high molecular weight and rigid structure. Some demerits of scleroglucan are its cost, ease of biodegradation, and poor filterability.

Hydroxymethycellulose (HEC) is a derivative of amorphous cellulose and non-ionic in nature. HEC has high molecular mass, rigid backbone structure, cheap in cost, non-toxic, and eco-friendly features suitable for uses as an ideal biopolymer. HEC is a shear resistant, salinity durable, and temperature tolerant biopolymer. HEC has some drawbacks like oxidation, biodegradation, and enzymatic degradation.

6.6 Biotechnological Upgradation of Produced Oil

Biotechnological upgradation of produced oil is industrial interest. This is due to the increase in concentration of sulfur in produced oil. Increased sulfur content in oil is a major environment concern. Biodesulfurization is a biological method for the removal of sulfur content in produced oil. Biodesulfurization was initially developed for the removal of inorganic (pyrite) and organic sulfur from coal by microorganisms. In petroleum, organic sulfur removal in transportation fuels is also a major concern. In crude oil, benzothiophene (BT) and dibenzothiophene (DBTs) are two main organosulfur compounds that are recalcitrance to removal by refinery process (e.g., hydrodesulfurization). DBT is the model compound for biodesulfurization research. Many microbes were investigated for their ability to utilize DBT as a sole sulfur source. Anaerobic desulfurization is less investigated, thus lack of anaerobic biochemistry and genetics of the desulfurization process are a major hurdle for commercial desulfurization process development. In the biodesulfurization process, there is low energy requirement, less generation of unwanted products, and low emission of sulfur containing compounds compared

to hydrodesulfurization. The biodesulfurization process is carried out at low pressure and mild temperature. In biodesulfurization, DBT and other sulfur compounds are transformed in harmless compounds by microbes. For increasing the efficiency of biodesulfurization process, genetic engineering technology has also been applied (Ma 2010).

Rhodococcus erythropolis has a high ability to remove sulfur from crude oil. This species is aerobic, chemoorganotrophic, gram negative, non-motile, and non-endospore forming. It also has a variety of catabolic enzymes, more than one plasmid. Thus, all these properties give greater biotransformation capability across a wide range of compounds. It utilizes the DBT as a source of sulfur, rather than a source of carbon and uses 4S pathway for DBT desulfurization. Other than *Rhodococcus, Agrobacterium, Mycobacterium, Gordonia, Nocardia, Sphingomonas, Stenotrophomonas, Sphingobacterium, Klebsiella, Pseudomonas, Arthrobacter*, and *Bacillus* species have shown capabilities for desulfurization of crude oil and oil products (Alkhalili et al. 2017).

Other than the desulfurization of produced crude oil, heavy oil to light oil conversion is also a bio-upgradation process for crude oil. This is now been developed as a new focus area by oil industries and petroleum microbiologist. Heavy crude (bitumen) is majorly produced by unconventional oil reservoir (e.g., Venezuela oil sands, Athabasca oil sands). This heavy oil is very viscous in nature and contains a high concentration of asphaltene, resins, nitrogen, and sulfur containing hetero-aromatics and several metals (nickel and vanadium). This heavy oil has a high production cost, difficult in transportation, and requires a conventional refining process (Leon and Kumar 2005). For transportation of heavy oil, solvent addition is needed for easier flowing in pipelines. The cost of solvent and vast heavy oil production require another method for decreasing its viscosity.

The upgradation of heavy oil is generally done by thermal cracking or by catalytic hydro conversion. But both processes have some common concern like been energy and cost intensive, less selective and environment reactive. Biological conversion could be an alternative process for the upgradation of heavy crude oil. Biological process is less severe and highly selective for specific reactions. This biological upgradation can be done by the microbial and enzymatic transformation process.

6.7 Intervention of Biotechnology in Unconventional Oil and Gas Production (Oil Sands and Shale Gas Fields)

Oil sands are heavy oil deposits that have a composition of sand, clay, and petroleum. These unconventional oil reserves are present in the Orinoco Oil Belt (Venezuela), Athabasca (Alberta, Canada), Olenik (Siberia, Russia), and Maya (Mexico) heavy oil sands. These reserves contain oil with high levels of bitumen. For the recovery of oil from such oil sands, very few biotechnological approaches are proposed. Therefore, biotechnological intervention can be applied on tailing ponds management. Oil sands tailing ponds are engineered dam and dyke, contain the waste by-product such as water, sand, slit, clay, and residual bitumen produced

during oil sand extraction and upgrading. Thus a proper management of tailing ponds is the main concern of oil sands operators.

Tailing ponds act as a good habitat for microbial growth and raise a major environmental concern. SRB and methanogens are easily grown in tailing ponds due to the availability of sulfate and anaerobic environment. Currently, oil sands mining companies are operating under a zero effluent discharge policy to the environment. In Canada alone, 1 billion m^3 tailings are stored in tailing ponds and cover approx. 220 km^2 area (Foght et al. 2017). For reclamation of land, some proposals related to placing tailing semi solids in the basin and capping with fresh water (end pit-lake) ecosystem or wetlands have been proposed. Other options available are placing de-watered tailing and covering with sand, soil, and vegetation for the generation of the boreal forest.

Potential biotechnological approaches employed for the remediation of tailing ponds are the aerobic treatment of froth treatment tailings. This leads to biodegradation of hydrocarbons prior to deposition in tailing ponds. This aerobic treatment also decreases the toxicity of ponds and also decreases methane emission. Another method employed is the in situ aerobic biological treatment of toxic organics (e.g., naphthenic acid). In this, partial oxidation of naphthenic acid is performed by ozonation, followed by the biodegradation process. Other approaches involves use of biofilm based bioreactors and algal based bio-treatment. Besides, microbial sequestration of produced gases (e.g., CH_4, CO_2) by methane oxidation is known to occur by native tailing ponds methanotrophs. Currently, none of the approaches is applied in pilot scale or in situ. While some of the approaches are in conceptual stages such as improving bitumen recovery by pretreatment using mEOR methods (uses of biosurfactant), uses of encapsulated microbes to remediate tailing ponds water, electricity generation by the construction of microbial fuel cells (Foght et al. 2017).

Shale gas, on the other hand, is a natural gas trapped within shale formations/ rocks. Shale rocks are clastic sedimentary rocks and formed from the mud, clay, and organic matter. Shale rocks porosity is very low (10–100 nanodarcies) and natural gas are trapped in the tiny pores. Initially, shale gas production is economical, but was not feasible due to the unviability of technology. At present, shale gas production is economically viable with the advancement of technology and improved methods. Shale gas is produced through horizontal drilling and hydraulic fracturing process. Major shale gas producing countries are China, USA, Canada, and Argentina. Shale gas production has some issues like leakage of extraction chemicals, high water requirement, and leakage of greenhouse gases during extraction and pollution due to the processing of natural gas. During the hydraulic fracturing, water based fluid is injected in well at high pressure to create cracks in shale rocks. This water based fluids contain water, diluted acids, biocides, breakers, corrosion inhibitors, friction reducers, gels, oxygen scavengers, proppant, scale inhibitors, and surfactant (Arthur and Layne 2008). Hydraulic fracturing fluid is susceptible to microbial growth. These microbes can be injected through fluids during drilling, drilling mud, and drilling water or indigenous to the shale gas field. To reduce microbial growth biocides are added in hydraulic fracturing fluids.

Among various microbes, acid producing and sulfate reducing bacteria induce corrosion in pipeline, while SRB create problem of souring. Growth of microbial biomass leads to well clogging due to biofilm formation, and subsequently inhibits the gas production. For better control of microbial growth, prior investigation of microbe types and concentration, carbon sources, nitrogen sources, electron acceptors, and growth limiting factors is required. The next generation sequencing tool is frequently used for knowing such microbial community composition in hydraulic fracturing fluids.

6.8 Conclusion

In oil field setup diverse microbial communities are present. In these microbial communities mainly, sulfate reducing bacteria, nitrate reducing bacteria, fermentative bacteria, iron reducing bacteria, iron oxidizing bacteria, and methanogens are dominated. Factors such as downhole reservoir temperature and pressure are extreme for the oil field microbial communities. In oil field system, oil organics are used as a carbon source, while inorganic compounds (such as sulfate, nitrate, and iron) are used as electron acceptor. In an oil reservoir, during oil biodegradation firstly higher oil components are converted into lower oil components by fermentative bacteria. These lower components are further used by sulfate or nitrate reducing bacteria. At the last stage, methanogens use one or two carbon compounds and converted into methane. Thus, due to microbial growth in oil field system, field operators face various problems such as microbial souring and biocorrosion, etc. The SRB is responsible for microbial souring in oil reservoir. To inhibit microbial souring various measures are taken by oil industries. The biotechnological approaches have great potential to control souring. Application of metabolic inhibitors and bacteriophage are the latest novel techniques to control souring with least environment impact and specifically target to problematic microbes only. Other than souring, different biotechnological approaches are applied in controlling microbial induced corrosion, EOR process, biodesulfurization, tailing ponds, and shale gas production. In mEOR, different microbial and plant origin biopolymers are applied to enhance oil production. Nanofluid with polymer is also applied as a novel strategy for the EOR process. For biodesulfurization, microbes and immobilized microbial cells are applied. For improving efficiency of desulfurization, recombinant DNA technology is becoming a good option. For reclamation of tailing pond affected land, aerobic treatment, biodegradation of oil components, and microbial sequestration are used. For upgradation of heavy oil, microbes can be used which utilize heavy oil components and convert it into lighter oil components. In shale gas fields, microbial souring, biocorrosion, and degradation of fracturing fluids are major problems. These problems are addressed by biocide injection in fracturing fluids. But prior to uses of biocide, microbial community composition should be known for the designing of appropriate control strategy.

6.9 Future Trends

Biotechnological intervention in oil industries is developed from the last three decades. Innovative biotechnology may offer a new way to reduce emission pathways in unconventional oil resources. New policies are aimed to maximize recovery from conventional and unconventional oil reserves. Production of fossil fuels from unconventional reserves is more expensive than conventional reserves. Therefore, there is a clear scope of less energy driven microbial process for recovering energy from these resources. Miscible flooding approaches can be applicable for heavy oil reserves (Shah et al. 2010). In situ catalytic upgrading of oil through Ni-, Co-, Mo-, and Pd-based catalyst is yet another purposed technique. Microbial deposited Pd nanoparticle has benefited over the physically prepared nanoparticles with respective to its application in in situ catalytic upgradation and oil recovery (Hart et al. 2016; Head and Gray 2016). Other than these possible intervention, enhanced energy recovery from residual oil, where secondary and tertiary recovery processes were no longer economical, is proposed. In this, methanogenic consortia convert alkanes to methane. This approach reduces the CO_2 emission (compared to oil and gas).

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Aquatic Microbial Oxygenic Phototrophs: A Short Treatise on Diverse Applications and the Future Biofuel Scenario

7

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Abstract

Man has relied upon microalgae ever since millennia. The importance of microalgal biotechnology as an exclusive niche in the industrial state of affairs is undeniably indisputable. Microalgae have been used to produce a wide variety of high value exploitable commercial products/metabolites such as antioxidants, carotenoids, vitamins, biomolecules (carbohydrates, proteins, and lipids), etc. Microalgae also hold great promise for the forthcoming biofuel industry. Microalgal biofuel are poised to be sustainable alternatives to conventional petro fuels; however, they need to overcome certain copious obstacles in order to compete in the international fuel market for an extensive commercial deployment. The scientific community is actively involved in research to establish microalgae as a biofuel podium. Progress made in this field is noteworthy, however, scientifically demanding and intellectually rigorous research seems to be the need of the hour. This article emphasizes on the non-energy and energy prospects of microalgal biomass with additional focus on the research gaps. This article aims to disseminate first-hand state-of-the-art information to help researchers, technocrats, venture capitalists, and policy makers in their futuristic endeavors pertaining to microalgal biotechnology.

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7.1 Introduction

Today's world is confronted with a global energy crisis. Adding to this energy crisis is the planetary emergency of global warming resultant from extensive use of fossil fuel usage. Declining geological reservoirs and the allied environmental issues have put the whole world at stake, whereby warranting global efforts for exploring clean and carbon neutral energy sources. The answer may lie well in our past, i.e. reliance on biomass as the source of energy. The biomass sources are renewable in nature, have a wider geographical distribution, and are environmentally benign. But the real question remains, which biomass we require to satisfy the escalating energy demand? Although time and again different researchers have rolled up the dice in favor of plant derived biomass, microbial biomass may have their own story of credibility. Aquatic Microbial Oxygenic Phototrophs (AMOPs) generally refer to algae, cyanobacteria, and diatoms. Among all these organisms algae (from here on microalgae for specificity) have been a topic of intense scientific focus for issues pertaining to energy crisis, energy security, and sustainable development.

Microalgae have garnered the attention of the scientific community as a biofuel feedstock (mostly biodiesel, bio-ethanol, pyrolytic bio-oil, and bio-hydrogen). They are promising biomass species that can serve as feedstock for the forthcoming biofuel industry. These wondrous microorganisms have been extensively investigated owing to their numerous salient attributes in comparison to terrestrial energy crops. Some of the salient prominent features of microalgae in this regard are:

1. The microalgal lipid content can easily be manipulated/adjusted by altering the respective growth media composition (Meher et al. 2006).
2. They rely exclusively on atmospheric carbon dioxide as the carbon source for their growth (Schenk et al. 2008).
3. The microalgal biomass doubling time during logarithmic phase may be normally as little as 3.5 h (Chisti 2007).
4. It is quite feasible to culture microalgae in waste as well as salty water (Schenk et al. 2008).
5. The intrinsic oil content in many species of microalgae exceeds 80% (by weight of dry biomass) (Chisti 2007).
6. Algae generally have superior rates of oil and biomass production when compared to conventional crops. This may be assigned to their simple cellular structure (Becker 1994).
7. Biomass from microalgae can be harvested almost all throughout the year (in batches). This ensures a consistent and incessant supply of oil (Schenk et al. 2008).
8. Various species of microalgae have been reported to produce different types of lipids, various complex oils and hydrocarbons (Metzger and Largeau 2005), which is much conducive in various biomass conversion processes.
9. It is possible to combine algal biofuel production with flue gas carbon dioxide alleviation, treatment of waste water, and subsequent production of high value bio-actives (Demirbas 2010).

10. Algae have been reported to produce 30–100 times more energy in a given area (per hectare) in comparison to terrestrial energy crops (Demirbas 2010).

The concept of biofuel production from microalgae is not new (Chisti 1980) but presently is being followed up critically because of soaring petro fuel prices and the planetary emergency of global warming associated with fossil fuel burning (Gavrilescu and Chisti 2005). The Arab embargo of the 1970s gave a new thrust to the field of microalgal biofuel. The Department of Energy's Office of Fuels Development (United States of America) launched the historic Aquatic Species Program (ASP) in 1978. This research project investigated biodiesel production from oleaginous algal isolates which were grown in ponds, and utilized waste carbon dioxide from coal fired power plants (Sheehan et al. 1998). The ASP was later on discontinued in 1996 owing to budget curbing, but however the status report of ASP serves as an excellent blueprint for initiating research in microalgal bioenergy. Despite the ASP out of the scenario research continued in this domain and now is well evident with tons of scientific literatures being available in the scientific repositories. There are substantial scientific efforts underway worldwide to investigate the probability of renewable biofuel production from various species of microalgae. The most important in this regard are methane (here in algal biomass is subjected to anaerobic digestion) (Spolaore et al. 2006), microalgal oil derived biodiesel (Chisti 2007), photo-biologically produced bio-hydrogen (Fedorov et al. 2005), and pyrolytic bio-oil produced by thermo-chemical conversion (Pan et al. 2010). Biofuel production from microalgal biomass is possible both in theory and practice and considering the present energy scenario is of global importance. Biofuel from microalgae indisputably appears to be promising in context of the existent energy shortage scenario; however, a critical impediment to their successful commercial implementation is the reasonably cheaper rates of petro fuels.

7.2 Non-energy Based Prospects

Apart from energy based products microalgae have other potential role such as in food additive, nutraceutical, biomedical domain, etc. Figure 7.1 shows the potential use of microalgae other than its role in energy based products. Microalgae are well-known rich source of high value bio-actives such as carotenoids, antioxidants, proteins, etc., which enhances the nutritional value of the food supplements (Hudek et al. 2014). Lutein which is a predominant carotenoid is found in many microalgae like *Muriellopsis* sp., *Scenedesmus almeriensis, Chlorella* sp., etc., and has a very high nutraceutical value (Guedes et al. 2011). Apart from Lutein, Astaxanthin and β-carotene are other carotenoids which have the potential nutraceutical values. Many microalgae like *Chlamydomonas*, *Chlorella*, *Oscillatoria*, *Scenedesmu*s, *Micractinium*, *Dunaliella, Spirulina*, and *Euglena* are well known for their high protein content which may be up to 50% of their dry weight (Islam et al. 2017). In Human muscle proteins; lysine, leucine, isoleucine, and valine amino acids are predominantly present, which account for nearly 35% of all amino acids.

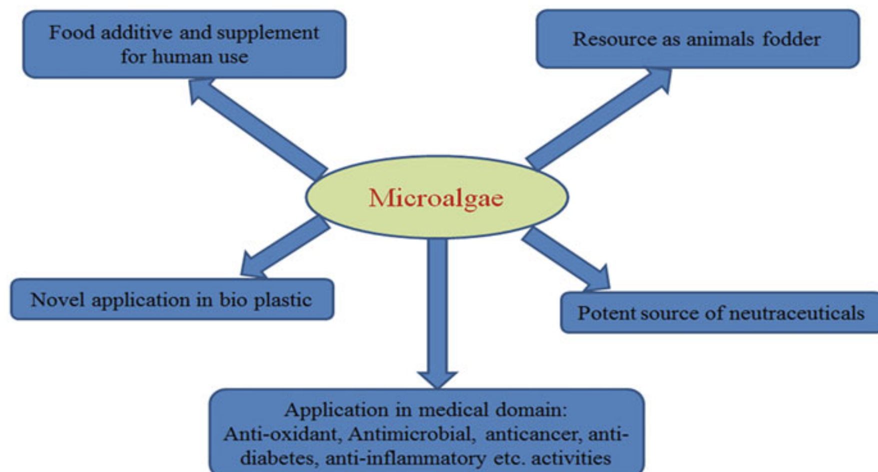

Fig. 7.1 Flowchart depicting non-energy based prospects from microalgae

Microalgae contain high amount of these amino acids. Thus they have proven application as dietary supplements to fulfill the requirement of proteins for all age groups (Dewapriya and Kim 2014). Minerals such as phosphorus, sodium, zinc, potassium, magnesium, manganese, iron, and calcium are very important for growth and nutrition in case of human and animals. Microalgae contain high amount of these minerals (Tokuşoglu 2003; Fabregas and Herrero 1990) which suggests its potential role as a nutraceutical agent. Several compounds isolated form microalgae have been reported for their potential use in biomedical domain such as naviculana polysaccharide isolated from *Navicula directa* reported as antiviral agent (Lee et al. 2006), compounds from *Phaeodactylum tricornutum* to induce leukemia cell death (Prestegard et al. 2009), marennine from *Haslea ostrearia* as antimicrobial agent (Gastineau et al. 2014), fucoxanthin and some microalgae as anti-inflammatory, anticancer, antidiabetic, and antimalarial activities (Peng et al. 2011; Lauritano et al. 2016), *Halidrys siliquosa* as anti-biofilm agent (Busetti et al. 2015). Several microalgal genera such as *Arthrospira, Chlorella, Dunaliella*, and *Haematococcus* are used as animal fodder which increases the production and quality of meat from livestock species such as pigs, ruminants, rabbits, and poultry (Madeira et al. 2017). Apart from all these biological activities, microalgae like *Chlorella, Spirulina, Nannochloropsis, Botryococcus braunii, Nannochloropsis gaditana* have been used to synthesize the bio plastics (Rahman and Miller 2017).

7.2.1 Microalgae from the Biotechnology Perspective

Microalgae are commercially used for human nutrition (*Chlorella*, *Spirulina*, *Dunaliella*) (Sathasivam et al. 2019), source of vitamins as dietary supplements (vitamin B_{12}, K_1) (Grossman 2016; Tarento et al. 2018), animal and aquatic feed

(Madeira et al. 2017; D'Este et al. 2017). A wide range of pigment molecules like carotenoids, astaxanthin which are antioxidants are produced by microalgae (Rammuni et al. 2019), polyunsaturated fatty acids (PUFAs), antimicrobial and anti-carcinogenic compounds (Kumar et al. 2019; Marrez et al. 2019), storage lipids or triacylglycerides (Xin et al. 2019), proteins, carbohydrates, and amino acids (Rizwan et al. 2018). Microalgae have garnered recent research attention particularly for bio-refinery (i.e., sustainable generation of biofuels along with high value metabolic co-products products by consolidated bio processing). In this context, oleaginous microalgae like *Nannochloropsis*, *Schizochytrium*, and *Botryococcus* (with oil content ranging from 20% to 60%, and up to 80%) have been widely explored for the biodiesel production (Bardhan et al. 2019) and other liquid biofuels (de Morais et al. 2019) (Table 7.1).

Genetic engineering tools have been used to modify microalgae for recombinant protein production, express genes to synthesize novel products, and increase the yield of natural value added products (Gangl et al. 2015). For example, *Chlamydomonas reinhardtii* was genetically engineered to produce xylitol (finds application in the food and confectionary industry as artificial sweetener) by integrating a xylose reductase gene from *Neurospora crassa* into its chloroplast genome (Pourmir et al. 2013). Furthermore, recombinant microalgae are promising cell factories for therapeutic protein production including antibodies, vaccines, and hormones (Gong et al. 2011). Recently, *Schizochytrium* sp. was genetically modified to produce a new antiviral vaccine against zika virus (Márquez-Escobar et al. 2018).

7.2.2 Microalgae from the Environmental Microbiology Perspective (Fig. 7.2)

Discharge of industrial effluent, municipal solid waste, agro-industrial waste water, pharmaceutical contaminants into freshwater systems has led to serious health issues and associated environmental hazards. In this context, microalgae mediated wastewater treatment and bioremediation of polluted contaminants has garnered recent research attention as it is solar-power driven, economically comprehensive, and sustainable strategy to mitigate these issues (Xiong et al. 2018). de Souza Leite et al. (2019) reported more than 90% removal of organic matter (in municipal and piggery wastewater) using *Chlorella sorokiniana.* Another study demonstrated the potential of using microalgae–bacteria consortium (two microalgal species, viz. *Desmodesmus* spp. and *Scenedesmus obliquus*) for the treatment of leachate/wastewater mixture along with microalgal biomass production having enhanced amount of accumulated lipids for the production of biodiesel (Hernández-García et al. 2019).

In addition to wastewater treatment, microalgae find immense application in soil as bio-fertilizers for nitrogen recovery (de Souza et al. 2019). Khan et al. 2019 demonstrated bio-refinery approach by integrating the phycoremediation potential of *Chlorella minutissima* with the subsequent production of biodiesel and organic manure. In terms of CO_2 capture process, microalgae has been found to have better (10–50 times more) CO_2 fixation ability than plants (Yadav and Sen 2017).

Table 7.1 Use of different microalgae in biotechnological application

Microalgae	Product/benefits	Application	References
Anabaena cylindrica	Vitamin K_1	Human nutrition	Tarento et al. (2018)
Spirulina platensis	Anti-inflammatory, antioxidant, and antihepatotoxic effects	Pharmaceutical, nutrition	Al-Qahtani and Binobead (2019)
Poterioochromonas malhamensis	Bio-active compounds (anti-bacterial)	Pharmaceutical	Schuelter et al. (2019)
Nannochloropsis oceanica	Biodiesel and aquaculture feed	Biofuels, shrimp feed	Ashour et al. (2019)
Thraustochytrium striatum	Astaxanthin	Nutraceuticals, cosmetics	Xiao et al. (2019)
Monoraphidium sp.	α-Linolenic acid	Infant formulas and nutritional supplements	Lin et al. (2018)
Tetradesmus obliquus	Galactooligosaccharides/β-galactosidase	Prebiotics/enzymes	Suwal et al. (2019)
Dictyosphaerium chlorelloides	Biopolymer (proteoglycan)	Food industry	Halaj et al. (2018)
Porphyridium cruentum	β-phycoerythrin	Fluorescent biomarker for diagnostics	Tran et al. (2019)
Chlorella pyrenoidosa, Scenedesmus sp., *Chlorococcum* sp.	Exopolysaccharides/antioxidant, antitumor activity	Pharmaceutical	Zhang et al. (2019)

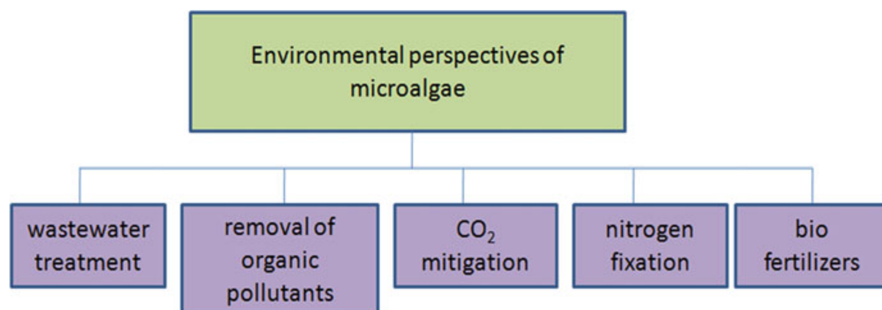

Fig. 7.2 Flowchart depicting different environmental perspective of microalgae

Table 7.2 Biotechnological applications of bio-active compounds from algae (Sharma and Sharma 2017; Priyadarshani and Biswajit 2012)

Sl. no.	Algal species	Different compounds	Uses
1	*Spirulina platensis*	Phycocyanins	Nutraceuticals, cosmetics
2	*Chlorella vulgaris*	Ascorbic acid	Health food, food supplement, food surrogate
3	*Haematococcus pluvialis*	Carotenoids, astaxanthin	Nutraceuticals, pharmaceuticals, additives
4	*Odontella aurita*	Fatty acids	Pharmaceuticals, cosmetics, baby food
5	*Porphyridium cruentum*	Polysaccharides	Pharmaceuticals, cosmetics
6	*Dunaliella salina*	Carotenoids	Nutraceuticals, food supplement, feed

Scenedesmus and *Neochloris* microalgal strains were found to be the most efficient as compared to different strains of microalgae or cyanobacteria for CO_2 capture process development (Sepulveda et al. 2019).

7.2.3 A Treatise on Commercial Applications of Microalgae

Presently, microalgae have received global interest owing to their prospective application in the renewable energy, nutraceutical, and biopharmaceutical sectors (Khan et al. 2018) (Table 7.2).

Microalgae have three essential features that can be capitalized for commercial and technical benefits (Priyadarshani and Biswajit 2012):

1. Microalgae are a highly diverse group from the genetical standpoint with an extensive array of biochemical and physiological characteristics; accordingly they are known to produce different altered and unusual fats, high value bio-active compounds, sugars, etc. (Richard and Bruce 1994).

Table 7.3 Useful substances present in microalgae (Priyadarshani and Biswajit 2012)

Pigments/carotenoids	β-carotene, astaxanthin, lutein, zeaxanthin, canthaxanthin, chlorophyll, phycocyanin, phycoerythrin, fucoxanthin
Antioxidants	Catalases, polyphenols, superoxide dismutase, tocopherols
Polyunsaturated fatty acids (PUFAs)	DHA(C22:6), EPA(C20:5), ARA(C20:4), GAL(C18:3)
Vitamins	A, B_1, B_6, B_{12}, C, E, biotin, riboflavin, nicotinic acid, pantothenate, folic acid
Other	Antimicrobial, antifungal, antiviral agents, toxins, amino acids, proteins, sterols, MAAs for light protection

2. They can cost-effectively assimilate the stable isotopes ^{13}C, ^{15}N, and ^{2}H into their biomass, and thereby into plentiful compounds they produce (Richard and Bruce 1994).
3. They represent a large, uncultivated group of organisms, and thus provide an almost accessible source of many valuable products (Richard and Bruce 1994).

Microalgae can be used as a prospective source of single-cell protein (SCP) (Priyadarshani and Biswajit 2012), a means of carbon sequestration (carbon dioxide) from stack gas, and can be utilized in sewage purification and for the production of biofuel. Microalgae have been projected as sunlight driven living-cell factories for biofuel production and several bio-chemicals used in food, aquaculture, poultry, and pharmaceutical establishments due to presence of different useful compounds (presented in Table 7.3).

7.2.4 Algae and Food

Global demand for algal foods (both micro and macroalgal) is escalating, and algae are increasingly being consumed for numerous practical benefits beyond the customary/traditional considerations of nourishment and health (Dawczynski et al. 2007). According to previous reports, algae are an exceedingly affluent source of vital and essential nutrients. They are a major source of human food in different nations, such as North & South America, France, Scotland, Ireland, Norway, Sweden, Germany, particularly in Asian countries like Japan, Peoples Republic of China, and Korea (Priyadarshani and Biswajit 2012; Kalau 2017). Even, microalgae are an affluent source of enzymes, protein, carbohydrates, and fiber. Additionally, numerous vitamins and minerals such as vitamin A_1 (Retinol), C (Ascorbic acid), B_1 (Thiamine), B_2 (Riboflavin), B_6 (Pyridoxine), Niacin (Nicotinic acid and a form of vitamin B_3), iodine, potassium, iron, magnesium, calcium, etc., are extensively found in microalgae (Priyadarshani and Biswajit 2012). Edible cyanobacteria (blue-green algae), such as *Spirulina*, *Nostoc*, and *Aphanizomenon* species have been used as food for millennia (Usharani et al. 2012). *Spirulina platensis*, a blue-green alga is increasingly gaining global reputation as a food supplement (being one of the most nutritious food source known to man) (Priyadarshani and Biswajit 2012).

Spirulina is an excellent source of proteins (Colla et al. 2007), pigments (Rangel-YaguiCde et al. 2004), poly unsaturated fatty acids (Sajilata et al. 2008) vitamins, and phenolics (Ogbonda et al. 2007).

7.2.5 Microalgae and Cosmetics

The cosmetic products from microalgae include mostly hair care and sunscreen products. Some typical microalgal species are well established in the skin care market such as *Mastocarpus stellatus, Chondrus crispus, Chlorella vulgaris, Ascophyllum nodosum, Dunaliella salina, Spirulina platensis, Nannochloropsis oculata*, *Alaria esculenta*, etc. (Priyadarshani and Biswajit 2012; Stolz and Obermayer 2005). The whole range of microalgal extracts can be largely found in skin and face care products (e.g., refreshing or re-generant care products, anti-aging cream, emollient, and as an anti-irritant in peelers (Stolz and Obermayer 2005). Some microalgal species are grossly utilized in companies in the skin care market. Some companies such as LVMH and Daniel Jouvance have landed in their personal microalgal cultivation systems. The various extracts from these species are an integral part of several cosmetics such as rejuvenating care products, anti-aging cream, sunscreen lotions, and hair care products (Sharma and Sharma 2017; Spolaore et al. 2006). There has been a soaring rise in procurement of various sunscreen products (which includes extracts from different species of microalgae) mainly due to increased public perception regarding skin cancer and photo-aging processes (Spolaore et al. 2006).

7.2.6 Microalgae and Food Colorant

Microalgal stains have marketable applications as aesthetic/cosmetic constituents and natural food colorants (Priyadarshani and Biswajit 2012). Various species of microalgae are known to produce an extensive array of colored pigments and consequently they are an appealing repertoire of natural colorants. Some microalgae cover substantial amounts of Carotene (besides β-carotene). β-carotene is used as a food colorant (major purpose in providing the yellow color to margarine), as a food additive to augment the color of fish flesh and eggs yolks, and to improve the well-being and productiveness/fertility of grain-fed cattle (Borowitzka and Borowitzka 1987).

7.2.7 As Bio-Fertilizers

Cyanobacteria (microalgae) have been known to play a cardinal role in maintenance and upsurge of fertility of soil, consequently enhancing crop growth (especially paddy), yield, and productivity as a natural bio-fertilizer (Song et al. 2005). From the cultivation perspective cyanobacteria in paddy cultivation is directly associated with

their ability to fix atmospheric nitrogen and other positive effects for plants and soil (Malik et al. 2001). Cyanobacterial species like *Tolypothrix, Anabaena, Nostoc*, etc., are efficient fixers of atmospheric nitrogen and are mainly used as inoculants for cultivation of paddy crops (both in upland and low land conditions) (Priyadarshani and Biswajit 2012). Jochum et al. 2018 reported that the two strains of N_2-fixing cyanobacteria (*Anabaena* sp. UTEX 2576, *Nostoc muscorum* UTEX 2209S), and a polyculture of *Chlorella vulgaris* (UTEX 2714) and *Scenedesmus dimorphus* (UTEX 1237) had improved the efficacy of microalgae based fertilizers in paddy growth with the help of using vertical semi-closed airlift photo-bioreactor (PBR) (Jochum et al. 2018). *Anabaena* in conjugation with the water fern *Azolla* contributes nitrogen (approximately 60 kg/ha/season) and also enhances soil quality with organic substances (Priyadarshani and Biswajit 2012).

7.3 Energy Prospects from Microalgae with Special Reference to Bioenergy

Speedily dwindling geological reservoirs, swelling energy demands, and increasing global concerns about the environment have compelled mankind to search for petro fuel substitutes. In this regard, numerous plant species have been investigated as potential source of biofuel; however, several lacunas associated with terrestrial energy crops/oil crops and lignocellulosic biofuel hinder their progression and popularity. On the global forefront, microalgae have been projected as a prime bioenergy source with the potential to replace conventional petro fuels (Chisti 2007). As discussed earlier in the introduction section, algae offer several advantages as compared to oil crops/energy crops and consequently have been extensively researched as a replacement for the predominant biofuel sources like sugarcane and corn. Several developed nations, emerging economies, and reputed companies like ExxonMobil, Sapphire Energy, Algenol, Solazyme, etc., have been already working towards the concept to commercialization of microalgal biofuel.

7.3.1 Biodiesel from Microalgae

Conventionally biofuel is mostly produced from plant oils such as corn, canola, soybean, rape seed, palm oil, *Jatropha*, *Pongamia*, coconut, ground nut, sunflower, mustard, etc. But none of these feedstocks can even pragmatically satisfy even a fraction of the present burgeoning need for energy (liquid biofuel). Furthermore, the conundrum of the Food Vs Fuel debate has geared up the quest for newer, sustainable, cost efficient, and environmentally benign feedstock for biodiesel (fatty acid methyl esters) production. A possible exception that may roll up the dice in favor of sustainability in near future is biodiesel production from microalgae. Over the past few decades microalgae have been the center of bioenergy research consideration. Microalgae today lie in the vanguard of bioenergy research as an emerging and promising feedstock for biodiesel production. Microalgal biodiesel research is now

one of the top notch research topics especially in the context of escalating petro fuel prices and climatic changes. Microalgae are characterized by an exceptionally speedy growth rate in comparison to plants/energy crops and additionally a significant proportion of their weight comprises oil. The microalgal oil following suitable extraction procedures can be reacted with an alcohol to get biodiesel which is renewable and environmental benign in nature. Also from the theoretical perspective, microalgae present strong candidature as a viable bioenergy feedstock for biodiesel production. The yield of oil from microalgae (on per unit area basis) is predicted to be 20,000–80,000 L/acre/year (Demirbas and Demirbas 2010). The theoretical yield is 7–31 times higher than palm oil (the next best crop for the production of biodiesel) (Demirbas and Demirbas 2010). Microalgae have been hypothesized to be the sole source of renewable biodiesel competent of meeting the international demand for liquid transportation fuels (Chisti 2007).

7.3.2 Bio-Oil from Microalgae

Biodiesel production from microalgae involves lipid extraction followed by transesterification. Following lipid extraction the microalgal de-oiled cake/remnants (low value biomasses refuse devoid of oil) are left. Finding suitable prospects for the microalgal de-oiled cake is one of the utmost challenges for the forthcoming microalgal bio-refineries (Ferrell and Sarisky-Reed 2010). Previously the microalgal de-oiled cakes were used as an aquaculture feed. But now with the energy crisis hitting the block, scientists are more concerned in finding suitable energy based options from the de-oiled cakes. One feasible alternative in this regard which would also be influential in reducing the economics of feedstock utility is pyrolysis of microalgal remnants to obtain renewable bio-oil and other value added products (bio-char and syn-gas). A few studies done in this direction by Pan et al. 2010, Wang et al. 2013 warrant the feasibility of pyrolytic bio-oil production from microalgal remnants.

In the wake of recent advancements in bioenergy research pyrolytic bio-oils have already gathered the attention of the scientific community in that they offer potential candidature not only as a chemical feedstock but also as a progressively attractive fuel option. However, it is interesting to note that there is dearth of scientific information about pyrolysis of direct microalgal biomass or its remnants in comparison to the pyrolysis of lignocellulosic biomass. A few studies have been conducted in this regard (Du et al. 2011; Miao et al. 2004; Miao and Wu 2004). Researchers have suggested that thermo-chemical conversion of de-oiled cake via pyrolysis can produce bio-oil which in some admiration is superior to bio-oil obtainable via pyrolysis of lignocellulosic biomass (Du et al. 2011, Miao et al. 2004, Miao and Wu 2004).

A major lacuna of microalgal biomass as a pyrolysis feedstock is the elevated nitrogen content in the bio-oil product. As per previous studies (Becker 2006) most of this nitrogen is present as protein in fast growing autotrophic microalgae. Additional nitrogenous ingredients of microalgae comprise nucleic acids (DNA and

RNA), chlorophyll, glucosamides, and cell wall materials though at reasonably low levels (<0.6 wt%) when compared to protein (10 wt%) (Devi et al. 1981; Becker 2006).

7.3.3 Bio-Ethanol from Microalgae

Bio-ethanol production from microalgae is not a new concept (although to a lesser extent in contrast to microalgal biodiesel). Currently, there have been incredible surges in research and development efforts to investigate the deployment of microalgae as a superior bioenergy feedstock for bio-ethanol production processes (Subhadra and Edwards 2010). The global interest in microalgae as a bio-ethanol feedstock is because they do not require arable land for cultivation and consequently do not contribute to the Food Vs Fuel debate. On to the contrary, existing bio-ethanol crops such as sugarcane, soybean, and corn contribute to the challenge of the Food Vs Fuel dispute.

Microalgae have been reported to store substantial fraction of carbohydrates in the form of glycogen, starch/cellulose, pentoses, and hexoses which can be converted into fermentable sugars via fermentation for bio-ethanol production (Wayman 1996). Bio-ethanol can be produced from microalgal biomass by utilizing amylolytic biocatalysts which facilitate starch hydrolysis and successive formation of fermentable sugars. Following fermentation of these sugars they can be distilled by means of distillation technology to obtain anhydrous bio-ethanol.

Although there is paucity of scientific literature with regard to bio-ethanol production from microalgae the process offers certain distinct advantages. Algal fermentation processes involve less energy intake and the procedure is much simple in contrast to production scheme for biodiesel (Singh and Gu 2010). Furthermore, carbon dioxide released by fermentation process can be recycled as a carbon supply for microalgae cultivation, thereby reducing greenhouse gas emissions (Singh and Gu 2010).

In today's scenario much of the scientific attention in microalgal biofuel research is concentrated in the biodiesel production from various species of oleaginous microalgae. A major production scheme for biodiesel production from oleaginous microalgae would generate enormous quantum of microalgal remnants/de-oiled cakes. Conversion of these microalgal remnants (chiefly comprising carbohydrates and proteins) into bio-ethanol may be a lucrative alternative in this regard. However, production of bio-ethanol from microalgae is at its infantile stage and warrants additional scientific investigations.

7.4 Research Gaps in Microalgal Biotechnology

Research gap literally exists in all the aspects of microalgal biotechnology. Microalgal biotechnology must enable us in getting products superior in quality, economically competitive (low at cost), and massive in scale. This is achievable only

if the existent research gaps are addressed properly. Research gaps need to be addressed for cost cutting in every step of the supply chain and commercially realize the full potential of microalgal biofuel and associated value added products. An integrated bio-refinery based approach is always preferable, where in the microalgal feedstock can be converted to biofuel in conjugation with a spectrum of other valuable products. The idea of zero waste bio-refineries is very popular in modern times, where in any waste generated in the production scheme automatically becomes the feedstock for the next product. Although significant progress has been achieved in the field of microalgal biotechnology, much more strenuous research is warranted to realize economically competitive algal biofuel and other value added products. Many subareas such as microalgal biology, strain improvement, microalgal cultivation systems (mostly mass culture approaches), process optimization, microalgal harvest and dewatering, new extraction technologies, genetically engineered strains (GMOs), biomass conversion, product recovery, co-product generation, value addition, fuel processing, economic analysis, etc., still remain the cardinal check points. However, addressing all these is beyond the scope of the present article. This article will consider only the life cycle assessment part. Although microalgae based biofuel production is emerging out as a top notch domain of research, there are many challenges and technical obstacles for massive commercialization of the fuel processing technologies. Microalgal biofuel production involves complicated cultivation, harvesting, dewatering, oil extraction, conversion, and purification steps. Adoption of suitable and efficient harvesting, extraction, or conversion technology is very crucial for an overall sustainable process economics (Shi et al. 2019).

Several methodologies for assessing environmental impacts and better sustainability for chemical process industry have been followed in recent years. Life cycle assessment (LCA) study among these is viewed as of paramount importance and imperative for microalgae based biofuel productions (Shi et al. 2019; Wang et al. 2011; Lardon et al. 2009). However, these analyses also give different results as biofuel production from microalgae largely relies on assumptions pertaining to algae cultivation methods, biomass yield and lipid content parameters, oil extraction methods and post treatment processes related to purification and upgradation (if any). For example, dewatering has been proposed as the most energy consuming and GHG emitting steps in an overall LCA of algae biodiesel production (Sander and Murthy 2010).

Another LCA results reported that flocculation had the lowest impact among three algae harvesting options, namely centrifugation, filtration, and flocculation/settling. The results of the study also showed chitosan and hexane as the most suitable, energy intensive flocculant and solvent, respectively (Brentner et al. 2011).

In a recent finding, "after modeling 160 pathways for combinations of different technologies of each process stage, the overall best-case scenario for well-to-wheel study was found to be flat-plate photo-bioreactor cultivation, chitosan flocculation, supercritical methanol combined extraction and transesterification, and energy recycle through recovery of biogas, from a LCA perspective, and total life cycle GHG emission amounts to 8.05 g CO_2 eq per MJ of biodiesel (Shi et al. 2019)." Although,

it is quite complicated to evaluate these manifold LCA results on harvesting and extraction unit procedures and subsequently resolve which technology leads to preferable environmental performance (Shi et al. 2019; Wang et al. 2011; Lardon et al. 2009; Sander and Murthy 2010; Brentner et al. 2011).

7.5 Concluding Remarks and Future Projections

The preceding decades have witnessed a major upsurge in microalgal biotechnology based research. Specific contributions to this pool of scientific knowledge are well evident with numerous scientific publications and patents from all around the globe. On the global vanguard there are numerous algal firms which produce and harvest algal biomass for varied applications (biofuels and value added products). Additionally, the number of startup companies attempting for commercial deployment of algal based biofuel is constantly in the rise. Algal biofuel undeniably offers a win–win situation, but still strenuous research is required for successful all round commercial deployment. A zero waste integrated microalgal bio-refinery based approach (where any waste generated becomes the feedstock for the next product) is likely to improvise the economic viability of algal biofuel. On a serious note much work needs to be realized in specific domains such as genetic and metabolic engineering of microalgal strains (strain improvement), growth and process optimization, microalgal biomass productivity, harvesting, bioreactor designing, enzymatic hydrolysis, lipid modulation, energy recycling, in expensive mass culture approaches, efficient and modern biomass conversion technologies, downstream processing, value addition (especially by-products), etc., for an enhanced inclusive understanding. Although, concept to commercialization of algal biofuel is only a matter of time, in the ensuing future algae is likely to play a paramount role in the international road transportation fuel mix. Nevertheless, it would also be interesting to see how algal biofuel would compete with new kids like hybrid cars in the near future where much progress has been made in the battery and energy efficiency technologies.

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Part V

Environmental Health

Bioinformatics: A New Insight Tool to Deal with Environment Management 8

Jinny Tomar

Abstract

The role of microbes in soil and water based biodegradation and cleaning of the environment has shown us the way to create and maintain a greener earth. The use of bioinformatics based tools supports the study of bioremediation and suggested promising results. Bioinformatics based applications allow to analyze data in silico and also enables to study the sequences, their gene–gene interactions, pathways involved, which directly indicate what kind of changes need to be incorporated to increase/induce the changes in the genetic makeup of selective plants and microbes to enhance the process of bioremediation.

8.1 Introduction

A lot has been said to protect the environment but in the current age of technology and rise in population. It is practically not advisable to stay on the old practices to manage the different kind of waste. The importance of introducing new age techniques to provide a better mode of safety is crucial and this cannot happen without introducing bioinformatics. Researchers these days are trying to focus on increasing the natural ability of different microorganisms to reinstate the environment.

When it comes to bioinformatics, it includes application of information technology, statistics, and biology to solve the various issues using in silico approach. The basics are crucial as the study will not only allow collection of data, storage in a systematic form, but will also emphasize on dissemination of the biological data relevant to our environment. Application of Bioinformatics in environmental studies is not limited to the study of genes which have been found effective in the process of bioremediation but also in identifying the role in the pathways involved, the

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gene–gene interaction as well as the protein–protein interactions study. This suggests that it is not just genomics, proteomics, but the study of transcriptome, and metabolome which is required to understand the entire mechanism and further take steps in the direction to achieve an ecologically stable environmental state.

With the advent of new technologies, industrialization has led to accumulation of hard metals and chemicals in the water sources, the soil, and underground water, air, making it a necessity to identify the customized or genetically altered microbes based bioremediation.

In order to understand the entire mechanism, bioinformatics based approach could be used to determine what exactly need to be known and how to retrieve that kind of data and further from which database, for example, a number of microorganisms have been identified which are capable of remediating pollutants but which database can provide such information, those details are not well known.

This entire study could be expressed in terms of gene ontology related studies, which includes identification of the molecular functions associated with the biological system, the cellular components playing important role in the same, and along with the understanding of the biological processes, making it easier to identify the role of specific processes in the entire system. Resequencing helps in better understanding of microorganism genome, the variation among the species, gene functions in the bacteria under selective criteria.

8.2 Bioinformatics and Its Application in Understanding Mechanism and Providing Suitable Solution to Pollution

The term Bioinformatics was first coined by Paulien Hogeweg and Ben Hesper in 1970 as “the study of information processes in biotic systems.” Bioinformatics based research and development deals with development of different softwares/tools as per user requirement. It also includes application of computational tools thereby leading to interpretation of different types of data based on nucleotide and/or amino acid sequence data and protein structure data.

Genomics and Proteomics are the two prominent research areas which have been able to receive lot of benefit due to the different tools of bioinformatics. On one hand, where after sequencing the contribution of bioinformatics in the form of biological databases and software is inevitable, similarly, on the other hand, protein based studies have not been easier without the different computational tools which had helped to identify specific proteins in the organism under study. Further these studies allowed identification of the superfamilies and the family of protein, conserved domains, motif, patterns, and profile. The protein–protein interactions, gene–protein network, different metabolic pathways can be studied with the help of bioinformatics based tools. Basically it can be said, that understanding of the biological systems can be done with the help of computational tools.

When it comes to study of the microbial genomes a number of steps can be performed for the analysis of the data. Few of such techniques include genome assembly, comparative analysis, study of evolutionary relationship, waste clean-up analysis. Genome assembly includes the designing of contigs, giving an idea of the

chromosome architect of the sequenced genomes by overlapping contigs. In this case the most common problem which arises is the presence of repeats, which are identical sequences of varying length and are found in different locations.

Various studies conducted include the understanding of the organisms, their fundamental processes enable to understand how the organism will react to the changes in the environment. This kind of studies can be done with the help of gene editing and other computational tools for constructing the metabolic models.

Signaling pathways may contribute a lot to the microbial population in a specified region at a given point of time. Study of microbial gene interactions using high throughput method has proved to overcome the limitations faced in culturing few microbes. Functional gene arrays have been found to play significant role in the study of genes responsible for degradation, metal resistance, antibiotic resistance, and so on (Nostrand et al. 2012).

Transcriptome analysis may enable to understand the impact and relationship between heat shock resistance, oxidative stress or nutrient starvation, heavy metal reduction, and even prediction and analysis of biogeochemical cycles of carbon and nitrogen (Bertin et al. 2008).

A large number of biological databanks have been developed in order to understand the complexity of biological networks relevant to the microorganisms involved in bioremediation process.

8.3 Bioremediation and Bioinformatics

Bioremediation is a process which helps in neutralizing the effect of waste in the environment. Different groups of microorganisms have been found to be effective in either neutralization or removal of the waste, by breaking down the organic matter they further allow decomposing the waste. It has been widely accepted and used as it is non-chemical based treatment and helps in recycling of the waste. This process has proven to be a savior as it not only helps in cleaning the environment but also helps in preventing us to have infections leading to degenerative diseases, cancer, respiratory disorders, cardiac issues, weakness of muscles, damage to kidney, liver, brain tissues due to accumulation of these toxics in the human body (Ojuederie and Babalola 2017).

On the basis of application it has been defined as: In situ, where the waste is treated at the site of origin, this also includes the onsite installation expenses, it further adds to the fact that proper control of the polluted site is difficult to manage. Ex situ remediation refers to the process where the contaminated waste is removed to a different site for effective treatment. This increases the cost of treatment due to the process of excavation (Azubuike et al. 2016).

Bioinformatics based approach have helped in the better understanding of the genomics, proteomics, metabolomics, and transcriptomics of the microbes involved to study and thereby enhance their degradative capabilities. With the help of different bioinformatics based tools it will be much easier to identify set of genes in respective microorganism which can mimic their action, as otherwise diversities

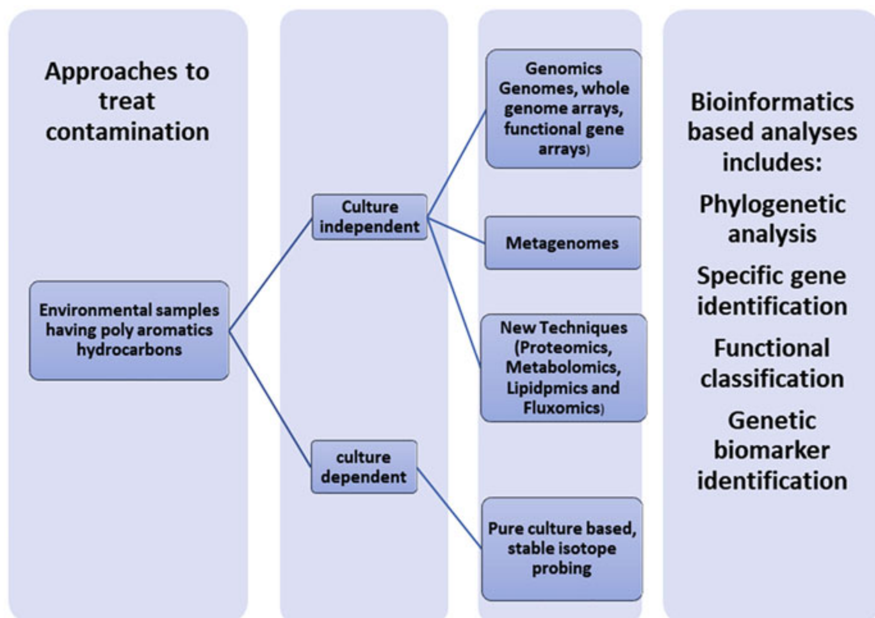

Fig. 8.1 Different approaches including bioinformatics as well as molecular biology based techniques to treat contamination

exist at all level of microbial remediation—differences in the isolates, their rate of metabolism, their adaptation, nutrient uptake, and so on. It has been an established fact that bioinformatics based study helps in reducing the time involved, reduces the number of steps by in silico based analysis, thereby further reducing the cost factor, making it a win–win situation. The same has been shown with the help of Fig. 8.1.

8.4 Different Types of Bioremediation

Techniques which have been developed for the process of bioremediation have been identified on the basis of factors like cost involved, characteristics of site under treatment, type and concentration of the pollutants, depth and degree of contamination, type of environment, environmental policies at that region.

This can be further categorized as either in situ approach or ex situ approach, where in situ is referred to as the treatment done at the site at which contamination/ pollutants are found and in case of ex situ the treatment is done at a different site, this implies the contaminant is excavated to a different location as shown in Fig. 8.2.

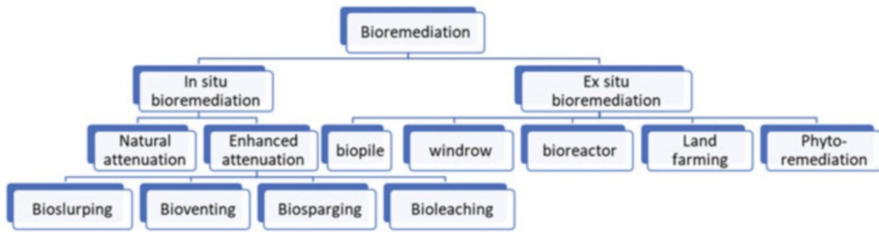

Fig. 8.2 The different bioremediation processes, highlighting the differences in terms of site of treatment of the pollutant

8.4.1 In Situ Bioremediation

8.4.1.1 Natural Attenuation

Monitored Natural Attenuation—It is considered as the most natural process as there is no human intervention, and the process solely relies on the pollutants and the microbial processes to breakdown the same. This intrinsic bioremediation model still needs to be monitored and risk management to be done accordingly.

8.4.1.2 Enhanced Attenuation

Bioventing

Air is allowed to pass through the waste, increasing the oxygen, leading to neutralization of the oxygen sensitive waste. Soil vapor extraction maximizes the vapor extraction, where the air flow is comparatively higher than bioventing.

Biosparging

This process is similar to bioventing, the difference lies in the fact that air is injected at the top layer, thereby pushing the volatile compounds movement to the upper zone to increase the rate of biodegradation.

Bioslurping

This technique has proved to be a combination of vacuum pumping, bioventing, and even soil vapor extraction process. This process is used in case of soil and water biodegradation for the treatment of volatile and semi-volatile waste.

Bioleaching

This could be defined as the process of extracting out the heavy metal and other kind of contamination with the help of living beings.

8.4.2 Ex situ Bioremediation

8.4.2.1 Phytoremediation

As the name indicates, with the help of plants, contaminants are kept closer to the plant body for physical, biological, and microbiological interactions, thereby decreasing the effect of waste from the water or soil. Important factors to be taken care of in this system includes—a fibrous root, which may be able to bind to pollutant based on the depth of the pollutant, the healthy shoot system for translocation from roots to shoot, and further the rate of transpiration depending upon the xylem flow and xylem sap.

Rhizofiltration/Rhizoremediation is a type of bioremediation in which plants and associated rhizospheric microbes are used to remove contamination of heavy metals in soil and water.

8.4.2.2 Land Farming

Depletion of the topmost layer of the soil, like in farming/tilling in order to introduce aeration, irrigation, and even addition of nutrients like nitrogen, phosphorus, and potassium. It has been categorized both under in situ (pollutant <1 m below ground surface) and ex situ (>1.7 m depth).

8.4.2.3 Bioreactor

It utilizes the use of specially designed containers to contain and treat the waste. A number of bioreactors are available like batch, fed-batch, sequencing batch, continuous, and multistage. The controlled parameters like temperature, pH, aeration rate, addition of nutrient as per requirement in timely fashion make it more efficient. It has also been seen that sewage sludge could be effectively utilized via this process and to improve the bioaugmentation process, even genetically modified organisms could be used, provided they are not allowed to reach the clean soil or water media, where the end product is released.

8.4.2.4 Composting

A natural way to dump the waste and wait for it to decompose, by breaking it down, although the process takes its own due course of time. Two prevalent and widely used aerobic technologies for composting include:

Biopiles

In case of Biopiles, the emphasis is more on regulation of the different processes involved like—aeration, irrigation, nutrient and leachation. The only difference between biopile and composting is addition of organic material to increase the temperature, thereby making favorable conditions for the growth of microorganisms. In this process aeration is achieved using plastic pipes, and this process can be done on large soil areas.

Windrows

The concept behind this process is turning of the pile periodically to enhance bioremediation in the polluted soil. It may not be the best technique for bioremediation of toxic waste. One of the best features is application of sewage sludge, vegetable sludge, and manure from cow; pigs, or even chicken can be used provided the material is available in bulk.

There are some other processes like bioaugmentation (process in which specific set of microorganisms are added to fasten the process of degradation in case of water or soil treatment) and biostimulation (use of microbes designed to remove contamination applied in a medium to the waste).

A number of factors contribute to the process of microbial bioremediation. The extent of effectiveness of bioremediation depends on the presence of microbial population, presence of oxygen, presence of moisture content, presence of nutrients like nitrogen, phosphorus, sulfur, and other nutrients for good microbial growth, presence of optimal temperature, and the result will be better in case of pH in the range of 6.5–7.5.

Challenges Faced During Bioremediation

It has been found that cadmium and lead are among the concern which as it has been found that they are difficult to degrade them using the process of bioremediation.

Impact of Bioremediation on the Nuclear Waste

In order to handle the nuclear waste the prominent approach includes usage of Bioreactor and the process of composting.

8.5 Role of Different Microorganism

The role of microorganisms is undeniable the natural way of degradation and cleaning of the environment (Rittmann 2006). It is this fact that led to focus on more research on the treatment of contaminated soil, water via the process of bioremediation. Efforts have been made so that maximum efficacy can be achieved by making few changes in the genetic makeup or may be introducing selective bacterial and fungal isolates to increase the pace of the degradation of hydrocarbons, aromatic compounds, sewage sludge, heavy metal contamination, industrial waste, petroleum related wastes, oil spills, and many more to be counted.

Among the major contaminants, polyaromatic hydrocarbons have been identified as toxic due to their carcinogenic nature and mutagenic effect. These PAHs have been found to contaminate the environment due to industrial activities, burning of plastic wastes, due to transportation, burning of fuels, forest fires, and so on. In air, PAHs are released in the form of particulate, in soil these are present as sediments, in water the pollutant is found accumulated in the aquatic life forms, making it more crucial to adapt effective measures like the process of bioremediation (Bisht et al. 2015; Chibwe et al. 2015).

Studies indicate that organic pollutants like trichloroethylene (TCE), atrazine (herbicide), trinitrotoluene (TNT), poly hydrocarbons, polychlorinated biphenyls, methyl tertiary butyl ether MTBE, and certain heavy metals like chromium, zinc, and even radioisotopes have been shown to give positive response to various bioremediation techniques.

The white rot fungal species which have been shown to be effective against organic/pesticide wastewater contamination using biopurification system (BPS) are *Phanerochaete chrysosporium, Trametes versicolor, Bjerkandera adusta*, *Pleurotus* sp., *Ceriporiopsis subvermispora*.

The contribution of species like *Coriolus versicolor, Hirschioporus larincinus, Inonotus hispidus, Phanerochaete chrysosporium, Phlebia tremellosa* in treatment of phenols obtained in treatment of colored dye pollution has already been well studied.

In case of wastewater treatment of oil spills, *Trichoderma harzianum, Mucor, Aspergillus* have proved to be effective source to start bioremediation process. *Cryptococcus sp., Pestalotiopsis palmarum* have been identified as efficient in treatment of crude oil contamination while *Aspergillus niger* and *Pseudomonas chrysosporium* play crucial role in bioremediation of petroleum and chlorpyrifos waste.

Fungi namely *Aspergillus, Pythium, Curvularia*, and *Acremonium* have been identified to play role in heavy metal treatment (Deshmukh et al. 2016).

The bacterial species contributing to hydrocarbon degradation in oil contaminated soil include *Acinetobacter*, *Micrococcus*, *Pseudomonas aeruginosa, Nocardia, Bacillus megaterium, Bacillus* sp., and *Corynebacterium*. (Abioye et al. 2012).

Role of *Pseudomonas putida* and *Aromatoleum aromaticum* cannot be ignored in toluene degradation, as petroleum hydrocarbons: aromatic hydrocarbons have been found in abundance in ground water, further contributing to the contamination (Grosbacher et al. 2018).

Role of metal ions like calcium, chromium, cobalt, copper, iron, manganese, nickel, potassium, sodium, and zinc is undeniable as they are considered as trace elements. Certain metals like silver, aluminum, cadmium, gold, lead, and mercury have no major biological role to play, and it has been found that in higher concentration these form complexes which induce toxic effects, thus making it more important to find natural way to undergo biotransformation (Olaniran et al. 2013).

Phytoremediation endophytes are those microorganisms—fungi and bacteria, which helps in the biotransformation of organic substances, which includes bioremediation of greenhouse gas pollutants—methane and carbon dioxide (Stępniewska and Kuźniar 2013). Endophytic microorganism can produce secondary metabolites which have shown to play a key role in the degradation of contaminants like phytoremediation of petroleum with the help of *Methylobacterium populum* sp., *Pseudomonas* sp. Endophytic fungi *Neotyphodium coenophialum* and *Neotyphodium uncinatum* have been found to reduce the concentration of polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbons (TPH) (Xia et al. 2014). *Enterobacter* sp. PDN3 has been shown to be effective in case of

trichloroethylene (TCE), phytoremediation of volatile contaminants. Methanotrophic endophytes prevalent in wetlands have been found to contribute to treating heavy metal contamination, organic contamination, and even greenhouse gases. This group involves microbes from *Burkholderia, Pseudomonas, Collimonas, Serratia*, and *Flavobacterium* spp. (Stępniewska and Kuźniar 2013).

Studies by Bisht and group, 2015 have emphasized that rhizoremediation technique has significant advantage in case of polyaromatic hydrocarbons related treatment. In this case few microorganisms like *Pseudomonas aeruginosa*, *Pseudomons fluorescens*, *Mycobacterium* spp., *Haemophilus* spp., *Rhodococcus* spp., *Paenibacillus* spp. have been found to be effective.

There are certain set of microorganisms which secrete biosurfactant, those compounds which acts as aid/enhancer of bioremediation process. A number of factors such as choice of surfactant, dose at the contamination site, interaction of the surfactant, biodegaradable nature of the surfactant decide the fate of surfactant enhancer bioremediation (SEB). These are *Mycobacterium* sp.*, Rhodococcus erythropolis, Pseudomonas* sp.*, Candida apicola*, *Acinetobacter calcoaceticus*, and *Thiobacillus thiooxidans* (Mohanty et al. 2013; Lawniczak et al. 2013).

A list of common environmental processes catalyzed by microbial guilds is provided in Tables 8.1 and 8.2 summarized an overview of the bioremediation potential of fungi.

This table has been introduced solely to highlight the significant role of different fungi in the process of bioremediation of different kind of wastes.

8.6 Gene Related Studies

Environmental genomics/ecological genomics/community genomics, or metagenomics is a field of science which enables to understand the genomes of different types of microbes present in the environment or community under study, further understanding the metaproteomics of the living system. This study plays a pivotal role in the process of bioremediation of pollutants, as it is these microorganisms are able to utilize these contaminants for their energy requirement. It is the genes present in these microorganisms which allow specific contaminant degradation as a carbon source or for energy purpose.

This field of science includes the study of genes, or orthologous set of genes and enables to relate the functionality of the microbial protein families with respect to particular metabolic functions. This implies in order to understand the entire functioning of the ecosystem, the link between the genomic data of the microbes, study of their phylogenetic relationship, along with the analysis of their interaction is significant, so that one can understand how the entire network functions (Morales and Holben 2011).

Metagenomics approach has helped in identification of novel gene products shown in Table 8.3, which have been found well suited for industrial purposes (Ojuederie and Babalola 2017).

Table 8.1 The list of common environmental processes catalyzed by microbial guilds (adapted from Perez-Garcia et al. 2016)

Catalytic microbial guild	Catalyzed environmental process	Service/application	Guild's model species	References
Aerobic heterotrophic bacteria	Organic carbon degradation (breakdown of suspended carbon to soluble carbon)	Organic matter removal from wastewater	Bacteroidetes α- and β-proteobacteria, *Acidovorax* spp., *Fermicutes* spp.	Wagner and Loy (2002), Wagner et al. (2002), Das et al. (2011)
	Organic carbon oxidation (soluble carbon to CO_2)	Organic matter removal from wastewater	Bacteroidetes α- and β-proteobacteria, *Acidovorax* spp., *Fermicutes* spp	Wagner and Loy (2002), Wagner et al. (2002), Das et al. (2011)
	Proteolysis (organic nitrogen to NH + 4)	Global nitrogen cycle, organic matter removal from wastewater	Bacteroidetes α- and β-proteobacteria, *Acidovorax* spp., *Fermicutes* spp.	Wagner and Loy (2002), Wagner et al. (2002), Das et al. (2011), Schreiber et al. (2012)
Heterotrophic denitrifiers	Denitrification (NO−3/ NO−2reduction to N2)	Global nitrogen cycle, biological nitrogen removal from wastewater	*Paracoccus denitrificans, Pseudomonas aeruginosa, Acidovorax* spp., α-, and β-proteobacteria	Ferguson, (1998), Brown (2010), Kraft et al. (2011), Schreiber et al. (2012)
Autotrophic nitrifiers, including both ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB)	Nitritation (NH + 4 oxidation to NO−2)	Global nitrogen cycle, nitrogen removal from wastewater	*Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosospira* spp.	Hooper (1991), Arp et al. (2002), Chain et al. (2003), Ferguson et al. (2007), Perez-Garcia et al. (2014b)
	Nitratation (NO−2 oxidation to NO−3)	Global nitrogen cycle, nitrogen removal from wastewater	*Nitrospira defluvii, Nitrobacter* spp.	Freitag and Bock (1990), Ferguson et al. (2007), Lücker et al. (2010), Schreiber et al. (2012)
	Nitrifier denitrification and hydroxylamine incomplete oxidation (production of NO and N2O)	Production and emission greenhouse and ozone depleting gases	*Nitrosomonas europaea, Nitrosomonas eutropha*	Shaw et al. (2006), Yu et al. (2010), Chandran et al. (2011), Schreiber et al. (2012)

Anaerobic ammonium oxidizers (ANAMMOX)	Ammonium oxidation to di-nitrogen gas (NH + 4 oxidation to N2)	Global nitrogen cycle, nitrogen removal from wastewater	*Kuenenia stuttgartiensis, Candidatus* Jettenia asiatica, *Brocadia anammoxidans*	Kuypers et al. (2003), Kuenen (2008), Hu et al. (2012)
Glycogen accumulating organisms (GAOs)	Anaerobic glycogen formation (carbon uptake and storage compound formation without phosphorus release)	Phosphorus removal from wastewater	*Micropruina glycogenica, Tetrasphaera* spp., *Amaricoccus* spp.	Seviour et al. (2003), de-Bashan and Bashan (2004), Martín et al. (2006), Wilmes et al. (2008)
Phosphate accumulating organisms (PAOs)	Anaerobic phosphorus release (hydrolysis of intracellular polyphosphates for carbon uptake and storage compound formation)	Phosphorus removal from wastewater	*Acinetobacter* spp., *Microlunatus phosphovorus, Clostridium* spp., *Candidatus* Accumulibacter phosphatis	Seviour et al. (2003), de-Bashan and Bashan (2004), Martín et al. (2006), Wilmes et al. (2008)
	Aerobic phosphorus uptake (storage compound degradation accompanied by soluble phosphorus uptake)	Phosphorus removal from wastewater	*Acinetobacter* spp., *Microlunatus phosphovorus, Candidatus* Accumulibacter phosphatis	Seviour et al. (2003), de-Bashan and Bashan (2004), Martín et al. (2006), Wilmes et al. (2008)
Polyhydroxyalkanoates (PHA) accumulating bacteria	Anaerobic formation of carbon storage compounds in form of polymers of the PHA family	Polyhydroxybutyrate (PHB) base bioplastic production	*Pseudomonas oleovorans, Alcaligenes eutrophus, Azotobacter vinelandii, Alcaligenes latus*	Batstone et al. (2003), Patnaik (2005), Dias et al. (2008)
Hydrogen producing acetogenic bacteria/archaea	Fermentation of higher organic acids to produce acetate, H2, and CO_2	Hydrogen and methane production	*Clostridium* spp., *Syntrophomonadaceae* spp., Bacteroidetes	Hatamoto et al. (2007), Rittmann et al. (2008), Khanal (2009a, b)
Autotrophic homoacetogenic bacteria	Syngas fermentation (use of hydrogen carbon monoxide and dioxide as carbon and energy source)	Ethanol, butanol, methane, and small chain fatty acid production	*Clostridium ljungdahlii*	Khanal (2009b), Abubackar et al. (2011)

(continued)

Table 8.1 (continued)

Catalytic microbial guild	Catalyzed environmental process	Service/application	Guild's model species	References
Heterotrophic homoacetogenic bacteria	Fermentation of higher organic acids and alcohols to produce acetate and CO_2	Methane production	Streptococcaceae and Enterobacteriaceae families, *Clostridium aceticum*, *Acetobacterium woodii*, and *Bacteroidetes* spp., *Clostridium* spp., *Lactobacillus* spp.	Hatamoto et al. (2007), Rittmann et al. (2008), Khanal (2009a, b)
Anaerobic methanogenic archaea	Acetotrophic conversion of acetate to methane	Methane production	*Methanosarcina* spp. and *Methanosaeta* spp.	Hatamoto et al. (2007), Rittmann et al. (2008), Khanal (2009a, b)
	Hydrogenotrophic conversion of carbon dioxide to methane	Methane production	*Methanosarcina* spp.	Hatamoto et al. (2007) Rittmann et al. (2008), Khanal (2009a, b)
Photo-autotrophs (microalgae/cyanobacteria)	Nutrient assimilation (soluble N & P assimilation to organic molecules)	Eutrophication of water bodies, nutrient removal from wastewater	*Chlamydomonas reinhardtii, Chlorella vulgaris, Spirulina platensis, Microcystis aeruginosa, Anabaena* spp., *Oscillatoria* spp., *Nostoc* spp.	de-Bashan and Bashan (2004, 2010), Perez-Garcia et al. (2010)
	Autotrophic CO_2 fixation (CO_2 fixation to biomass)	Global carbon cycle, biomass formation, CO_2 sequestration	*Chlamydomonas reinhardtii, Chlorella* spp., *Spirulina platensis, Microcystis aeruginosa, Scenedesmus obliquus, Nannochloropsis* spp.	Das et al. (2011), Cheirsilp and Torpee (2012), Girard et al. (2014), Wu et al. (2015)
	Autotrophic and heterotrophic lipid, starch and pigments production	Biofuels and valuable chemical production	*Chlorella vulgaris, Chlorella prototecoides*	de-Bashan et al. (2002), Perez-Garcia et al. (2011), Choix et al. (2012a, b), Perez-Garcia and Bashan (2015)

	Production of nitrous and nitrous oxides	Production and emission of greenhouse and ozone depleting gases	*Chlorella vulgaris*	Guieysse et al. (2013), Alcántara et al. (2015)
	Synthesis of exo-polymers	Bio-absorption of organic compounds and pollutants	*Chlamydomonas reinhardtii, Chlorella vulgaris, Spirulina platensis*	Markou and Georgakakis (2011), Subashchandrabose et al. (2013)
Cyanobacteria	Production and realization of secondary metabolites and toxic organic compounds (microcystin, nodularin, cylindrospermopsin, among others)	Self-population an grazer organism control	*Microcystis aeruginosa, Anabaena* spp., *Oscillatoria* spp., *Nostoc* spp.	Welker and Von Döhren (2006), Yadav et al. (2009), Kaplan et al., (2012), Dittmann et al. (2013), Neilan et al. (2013)
Dissimilatory metal-reducing bacteria.	Anaerobic Fe^{3+} reduction to Fe^{2+} (reduction of insoluble iron to soluble form)	Global iron cycle, bioremediation of metallic pollutants in soil and groundwater	*Geobacter metallireducens, Geobacter sulfurreducens, Albidoferax ferrireducens, Shewanella putrefaciens*	Lovley and Coates (1997), Malik (2004), Gadd (2010), Melton et al. (2014)
	Anaerobic Mn^{4+} reduction to Mn^{2+} (reduction of insoluble iron to soluble form)	Global iron cycle, bioremediation of metallic pollutants in soil and groundwater	*Geobacter metallireducens, Geobacter sulfurreducens, Albidoferax ferrireducens, Shewanella putrefaciens*	Lovley and Coates (1997), Malik (2004), Gadd (2010), Melton et al. (2014)
	Anaerobic As^{5+} reduction to As^{3+} (reduction of insoluble arsenic to soluble)	Bioremediation of metallic pollutants in soil	*Geospirillum arsenophilus, Geospirillum barnseii, Chrysiogenes arsenatis, Sulfurospirillum* strain NP4	Lovley and Coates (1997), Malik (2004) Lear et al. (2007), Gadd (2010)
	Aerobic Hg^{2+} reduction to Hg^{0}(reduction of soluble mercury to volatile form)	Bioremediation of metallic pollutants in soil and water	*Pseudomonas* spp.	Lovley and Coates (1997)
	Anaerobic U^{6+} reduction to U^{4+}(reduction of soluble uranium to insoluble form)	Soil bioremediation of radioactive pollutants	*Thiobacillus thiooxidans, Rhodoferax ferrireducens, Geobacter sulfurreducens, Shewanella putrefaciens, Desulfotomaculum reducens*	Lovley and Coates (1997), Malik (2004), Gadd (2010)

(continued)

Table 8.1 (continued)

Catalytic microbial guild	Catalyzed environmental process	Service/application	Guild's model species	References
	Anaerobic Tc^{4+} reduction to Tc^{7+} (reduction of soluble technetium to poorly soluble form)	Soil bioremediation of radioactive pollutants	*Geobacter* spp.	Lear et al. (2010)
	Anaerobic and aerobic Cr^{6+} reduction to Cr^{3+} (reduction of soluble chromium to insoluble form)	Bioremediation of metallic pollutants in soil and water	*Pseudomonas* spp., *Achromobacter eurydice, Desulfovibrio vulgaris, Bacillus* spp., *Desulfotomaculum reducens*	Wang and Shen (1995), Lovley and Coates (1997), Malik (2004), Gadd (2010)
Heavy metal resistant microbes	Heavy metal (Cu, Zn, Ni, Cd, Pb, Hg) immobilization by biosorption, bioaccumulation, biochelation	Bioremediation of metallic pollutants in soil and water	*Alcaligenes eutrophus, Alcaligenes xylosoxidans, Stenotrophomonas* sp., *Ralstonia eutropha, Staphylococcus*sp., *Pseudomonas syringae*	Lovley and Coates (1997), Diels et al. (1999), Malik (2004), Gadd (2010), Edwards and Kjellerup (2013)
Dissimilatory sulfate reducing bacteria	Anaerobic SO2–4 reduction to H2S (reduction of soluble and insoluble sulfur to volatile form)	Global sulfur cycle, treatment of sulfur and sulfate contaminated groundwater and industrial wastewater	*Desulfovibrio* spp., *Thermodesulfovibrio yellowstonii, Archaeoglobus* spp., *Desulfatibacillum* spp. *Desulfothermus* spp., *Desulfotomaculum reducens*	Lovley and Coates (1997), Malik (2004), Gadd (2010), Pereira et al. (2011), Hao et al. (2014)
Sulfur-oxidizing bacteria	Chemolithotrophic H2S, S^0oxidation to SO2–4 (reduction of soluble and insoluble sulfur to volatile form)	Global sulfur cycle, bioremediation of sulfur pollutants in water	*Beggiatoa* spp., *Thiobacillus novellus, Sulfolobus* spp., Purple and green sulfur-oxidizing bacteria	Lovley and Coates (1997), Kappler et al. (2000), Malik (2004), Gadd (2010), Pokorna and Zabranska (2015)

Iron oxidizing bacteria	Chemolithotrophic Fe^{2+} oxidation to Fe^{3+} (oxidation of soluble iron to insoluble form)	Global iron cycle, bioremediation of metallic pollutants in water	*Leptospirillum ferrooxidans, Acidithiobacillus ferrooxidans, Sulfobacillus thermosulfidooxidans*	Lovley and Coates (1997), Malik (2004), Gadd (2010)
Ectomycorrhizal fungi	Filamentous (hyphae) extension of plant root systems (do not penetrate plant root cells)	Enhance plant acquisition of nitrogen, minerals, and water	*Russula xerampelina, Amanita franchetii, Suillus bovinus*	Gardes and Bruns (1996), Chalot and Brun (1998), Reid and Greene (2012)
Arbuscular mycorrhizae fungi	Filamentous (hyphae) extension of plant root systems (penetrate plant root cells)	Enhance plant acquisition of nutrients, minerals, and water	*Rhizophagus irregularis, Piriformospora indica*	Reid and Greene (2012)
Endophytic fungi	Fungi–plant symbiotic production of bioactive compounds	Pathogen and predator resistance	Clavicipitaceae family	Reid and Greene (2012)
Lignocellulosic fungi	Lignin degradation to soluble carbohydrates mediated by peroxidases and laccase	Global carbon cycle, lignocellulosic biomass degradation, biofuel production, bio-refining of valuable chemicals	*Phanerochaete chrysosporium, Pleurotus* spp.*, Trametes versicolor, Phanerochaete chrysosporium*	Bugg et al. (2011), Harms et al. (2011)
	Organic pollutant degradation to harmless compounds mediated by peroxidase, laccase and cytochromes	Organic pollutant degradation, bioremediation	*Gloeophyllum* spp., *Trabeum* spp., *Gliocladium virens, Trametes versicolor, Phanerochaete chrysosporium, Candida* spp	Keller et al. (2005), Bugg et al. (2011), Harms et al. (2011), Lah et al. (2011), Margot et al. (2013)
Recalcitrant pollutant degrading bacteria	Organic pollutant degradation to harmless compounds mediated by peroxidase, laccase, and cytochromes	Organic pollutant degradation (pesticides, pharmaceuticals, agrochemicals, industrial waste chemicals, oil, and petrochemicals)	*Pseudomonas* spp., *Streptomyces* spp., *Desulfovibrio* spp., *Brevundimonas diminuta*	Díaz (2004), Head et al. (2006), Singh (2009), Guazzaroni and Ferrer (2011), Nikel et al. (2014)

(continued)

Table 8.1 (continued)

Catalytic microbial guild	Catalyzed environmental process	Service/application	Guild's model species	References
Plant growth promoting bacteria (PGPB)	Diazotrophic nitrogen fixation (di-nitrogen gas conversion to ammonia, which is available for plant assimilation)	Global nitrogen cycle, increase biomass production yields of plants or microalgae	*Azospirillum brasilense, Azospirillum lipoferum, Bacillus pumilus, Azoarcus* sp., *Rhizobium leguminosarum*	Hartmann and Bashan (2009), Hernandez et al. (2009), Reid and Greene (2012)
Plant and microalgae promoting bacteria	Phytohormone production (indole-3-acetic acid and gibberellin production)	Increase of starch formation, and ammonium and phosphate uptake by microalgae	*Azospirillum brasilense, Bacillus pumilus*	de-Bashan et al. (2002, 2005, 2011), Choix et al. (2012a, 2014), Meza et al. (2015a, b)

Table 8.2 Overview of the bioremediation potential of fungi (adapted from Deshmukh et al. 2016)

Compound	Fungi	References
POPs		
Polychlorinated biphenyls	*Doratomyces nanus*, *D. purpureofuscus*, *D. verrucisporus*, *Myceliophthora thermophila*, *Phoma eupyrena*, and *Thermoascus crustaceus*	Mouhamadou et al. (2013)
	Aspergillus niger	Marco-Urrea et al. (2015)
Polychlorinated dibenzofurans	White rot fungi	Wu et al. (2013)
	Phanerochaete sordida	Turlo (2014)
Phenylurea herbicide diuron	*Mortierella*	Ellegaard-Jensen et al. (2013)
Textile dye decolourization	*Aspergillus niger*, *A. foetidus*, *T. viride*,	Jebapriya and Gnanadoss (2013)
	A. sojae, *Geotrichum candidum*,	
	Penicillium sp., *Pycnoporus cinnabarinus*	
	Trichoderma sp.	
	White rot fungi	Ma et al. (2014)
	Bjerkandera adusta, *Ceriporia metamorphosa*,	
	Ganoderma sp.	
Petroleum products		
Crude oil	*A. niger*, *Rhizopus* sp., *Candida* sp.,	Damisa et al. (2013)
	Penicillium sp., *Mucor* sp.	
Gasoline	*Exophiala xenobiotica*	Isola et al. (2013)
Bleached kraft pulp mill effluent	*Rhizopus oryzae or Pleurotus sajor caju*	Duarte et al. (2013)
Effluent from leather tanning	*Aspergillus flavus*, *Aspergillus* sp. *and A. niger*	Bennet et al. (2013)
	Aspergillus jegita	Reya et al. (2013)
PAH		
Diphenyl ether	White rot fungi	Wu et al. (2013)
	Pleurotus ostreatus	Rosales et al. (2013)
	Trametes versicolor	
Anthracene	*Armillaria* sp.	Hadibarata et al. (2013a, b)
Naphthalene	White rot fungi	Hadibarata et al. (2013a, b)
	Pleurotus eryngii	
PPCP		
Caffeine	*Chrysosporium keratinophilum*, *Gliocladium roseum*, *Fusarium solani*, *A. restrictus*, *Penicillium, and Stemphylium*	Nayak et al. (2013)
Citalopram, fluoxetine, sulfamethoxazole	*Bjerkandera* sp. R1, *Bjerkandera adusta and Phanerochaete chrysosporium*	Rodarte-Morales et al. (2011)
Fungicide		
Metalaxyl and Folpet	*Gongronella* sp. and *R. stolonifer*	Martins et al. (2013)

(continued)

Table 8.2 (continued)

Compound	Fungi	References
Pesticide		
Chlorinated hydrocarbons: Heptachlor	*P. ostreatus*	Purnomo et al. (2013)
chlorpyrifos	*Aspergillus terreus*	Silambarasan and Abraham (2013)
Heavy metals	*Aspergillus*, *Curvularia*, *Acremonium*, *Pythyme*	Akhtar et al. (2013)
	Aspergillus flavus	Kurniati et al. (2014)

8.7 Major Pathways

As we know that the process of bioremediation can be executed either through the process of biostimulation, bioaugmentation or simply natural attenuation. This has been seen in case of not only industrial waste, petroleum waste, or even treating plastic waste.

Petroleum waste also contributes towards wastewater, the presence of phenols and phenolic compounds cause serious damage to the environment. Biological treatment which include green process treatment with the help of bacteria like *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Alcaligenes* work on the concept of aerobic degradation of phenolic compounds which happens when hydroxylation of the ring occurs at the adjacent carbon atom, this is followed by cleavage of catecholics using ortho cleavage using 1, 2-dioxygenase enzyme or meta cleavage pathway using 2, 3-dioxygenase enzyme.

Aniline is one of the toxic cancer causing organic compounds which is used in paints, pesticides, herbicides, plastics, and many more. Some of the bacterial species like *pseudomonas*, *acinetobacter*, *rhodococcus* are able to utilize aniline for carbon and nitrogen source. It has been found that two different pathways have been utilized for aniline/aminobenzene degradation, which include Beta ketoadipate pathway. Beta ketoadipate, gentisate pathway, and extradiol cleavage pathways have been identified to play major role in the degradation of aniline, mono cyclic aromatic compounds, hydroxybenzoate, toluene, xylenes, and salicylate (Lee et al. 2016).

It has already been seen in case of *Aspergillus nidulans* that degradation of mono chloro phenols makes use of 4-chlorocatechol and 3-cholorocatechol pathways (Martins et al. 2014).

Another important pathway based on carbon as source of energy utilization has been noticed in case of aerobic methanotrophs, which have been using ribulose monophosphate (RuMP) pathway and serine pathway (Ross and Rosenzweig 2016).

It has been seen that the microbial degradation of dioxane makes use of two distinct pathways—metabloic (most common) and cometabolic (several monooxygenase are involved—MMO, PMO, PHE, TOL, RMO, etc.).

Table 8.3 List of genes identified by various sources and found to contribute to bioremediation (Gedalanga et al. 2014)

Name of the gene	Specific heavy metal bioremediation	Citation
(*mer* gene cluster) *mer*A, *mer*T, *mer*P, *mer*F, *mer*R and *mer*D	Removal of mercury, Hg contaminant	Zheng et al. (2018)
ArsM, aioA, aioB, arxA, arxB/arxB2, aioS/arxR, aioX/arX, moeA, arxC, arxD, arsH, arrA, arrB, arrC, arrD, arrS, arsC, ACR1, ACR2, arrR/arsR, arsI, arsB, arsA, arsD, arsC, Ycflp, ACR3, arsP, arsJ, arsO, arsT, pgpA	Arsenic (As)m treatment	
pheA, *phe*B, *phe*C, *phe*D, *phe*R	Phenol catabolic genes	
alk gene (*alk*B, *alk*B1, *alk*B2, *alk*M)	Alkane degradation	van Beilen et al. (2002), Wang and Shao (2012)
sMMO	Methane degeneration	Ross and Rosenzweig (2016)
P450–1	Poly aromatic hydrocarbons	Schneiker et al. (2006)
P450–2	Poly aromatic hydrocarbons	Schneiker et al. (2006)
P450–3	Poly aromatic hydrocarbons	Schneiker et al. (2006)
Pcs	Bioremediation of non-toxic essential heavy metals	Shine et al. (2015)
yiLac	Phenolic compounds	Deshmukh et al. (2016)
pmoA	Trichloroethylene degradation	Shukla et al. (2009)
ethB	Transformation of methyl-ter-butyl ether	Jechalke et al. (2011)
pMMO	Dioxane degradation	Ross and Rosenzweig (2016)
Dxmo	Dioxane degradation	Ross and Rosenzweig (2016)
thmB	Tetrahydrofuram	Thiemer et al. (2003)
thmH	Dioxane degradation	Kampfer et al. (2006)
gsh1, gsh2	Cadmium tolerance	Liu et al. (2015)
Tdn	Aniline degradation	Lee et al. (2016)
pcaG, pcaH, pcaB, pcaC, pcaD	Hydroxybenzoate degradation	Lee et al. (2016)
nagG, nagH, nagI, nagL, nagK	Salicylate degradation	Lee et al. (2016)

So, basically it can be said that the carbon assimilation pathways, membrane morphology, presence of specific genes guide a cell to uptake specific pathway to perform the bio transformation process.

8.8 Biological Databases Relevant to Environmental Studies

There are a number of biological databases which have been able to provide information about the metabolic pathways, about the bio geochemical pathways which include various levels of microbial interactions among the different microorganisms (Lovely 2003; Perez-Garcia et al. 2014a). It will also be able to define the link between the sequence and the annotated genome using tools like Pathway Tools, which allow prediction of metabolic network with the help of Pathway/Genome Database (PGDB).

8.8.1 BioSurfDb

This is one databank providing information about different microorganisms, metagenomes, biodegradation related genes, specific proteins, and even the pathways associated with the same, it also includes the number of experiments with pollutants and their respective treatments. This curated database also provides information about the biosurfactant: name, class, and also references related to it (http://www.biosurfdb.org/#/getstarted).

8.8.2 MetaCyc

Databases like MetaCyc (metacyc.org) allow non-redundant databases, which contains information like metabolic pathways and even the respective enzymes involved. Kyoto Encyclopedia of Genes and Genomes (KEGG) also provides information giving interrelation of genes and genomes, with the number of enzymes, reactions, chemical compounds.

8.8.3 MetaRouter

This allows one to have dataset providing relevant information in context to bioremediation. It provides information in terms of enzymes involved, compounds, microorganisms, and the framework in which it is involved (Pazos et al. 2005).

8.8.4 Biodegradation Probability Program (BIODEG)

BIODEG enables to calculate the rate of biodegradation using microorganisms. This software gives the probability of rapid degradation under aerobic conditions.

Chemical degradation is predicted based on the chemical structure using SMILES notation (https://cfpub.epa.gov/si/si_public_record_Report.cfm?Lab=&dirEntryID=2895).

8.8.5 US Geological Survey Group

This has been able to provide tools for improving environmental health and its analysis. Among the tools, Natural Attenuation Software (NAS) for determining the sites which need cleaning for ground water contamination. It is a user friendly software, which provides timely monitoring of natural resources, considering the type and number of contaminants, also including the different solvents like chlorinated ethenes and petroleum hydrocarbons (https://toxics.usgs.gov/highlights/nas_2.2.0/).

8.8.6 CRAFT

Another major software CRAFT which helps by assessing the toxicity, hazards, and risk assessment associated at a specific area to evaluate chemical reactivity, persistence, biodegradation of chemical compounds in the environment (https://www.mn-am.com/products/craft).

8.8.7 BIOMOC

This is also a two dimensional model which helps in simulation of rate of biological transformation. This model includes calculation based on Monod kinetics, competitive–non-competitive and Haldane inhibitions. This model can be termed as an advanced/extended version of the US Geological survey Method of Characteristics (MOC) flow and transport model (https://water.usgs.gov/cgi-bin/man_wrdapp?biomoc).

8.8.8 EAWAG-BBD

Another category of biological database providing information about microbial biocatalytic reactions and biodegradation pathways is EAWAG-BBD, which provides data under two broader categories: Reactions and pathways. Its feature like Pathways prediction System helps in prediction of microbial catabolic reactions using sub structure, Predict BT system is based on the University of Minnesota Predictive Biodegradation Project which tries to predict the fate of new chemicals in the environment and the Biochemical Periodic Table gives a colored representation of the elements present in the periodic table with respect to their role in environment (http://eawag-bbd.ethz.ch/).

8.8.9 Clean-Up

United states Environmental Protection Agency has a database, Clean-Up Information which can help in innovative treatment of contaminated site and management of waste remediation (https://clu-in.org/techfocus/default.focus/sec/Bioremediation/cat/Overview/).

8.8.10 HSDB

A National Library of Medicine (NLM) initiative which is based on providing information about the toxic nature of chemicals, impact on human health, ways to introduce industrial hygiene, application of nanomaterials can be seen in this databank: Hazardous Substances Databank (HSDB) (https://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB) (Zhu et al. 2017).

8.8.11 TOXNET

Other than this, another major resource for safeguarding environmental health and resources is known as Environmental Health, Toxicology & Chemical Information, which has different resources like TOXNET and Toxicology Subset (https://envirotoxinfo.nlm.nih.gov/).

8.8.12 ToxTown

It has emerged as a database providing information about the chemicals and contaminants, various sources through which we can be exposed to such chemicals and also how the different diseases and medical conditions are even related to the environmental factors (https://toxtown.nlm.nih.gov/diseases-and-conditions).

8.9 Conclusion

The focus of this study is to make us understand the role of protecting environment which has been contaminated due to excessive using of chemicals in one or the other form. The significance of plant/microbe based mode to remediate waste generated both at the level of water and soil (based on biological mechanisms) can be done by making use of processes like biosorption, phytoremediation.

The need is to make sure that we are able to provide and maintain such conditions like optimum temperature, pH, high nutrient concentration which will naturally help in the process of degradation of the heavy metals and the toxic chemicals. Thus emphasis should be to identify the nature of toxic chemicals accumulated followed by providing adequate environment to the specific plants and rhizospheric microbial

species to enact the process of phytoextraction. If there is a need felt to introduce Genetically Modified Organisms (GMO), then proper safety measures should be taken care.

The use of bioinformatics has been able to provide, in silico based assumptions, making it more convenient to analyze how and which specific genes need to be modified/upgraded in order to have effective bioremediation process. There is still lot to learn about the different metabolic pathways favoring bioremediation in the microorganisms at grass root level. Thus it could be said that computational biology along with synthetic biology can bring the much desired boom required for creating, developing a whole class of microbes, which have the ability to degrade and remove all sort of accumulated waste chemicals. This can be achieved if we are able to merge metagenomics approach as well gene editing to help the world by removing the waste in the most appropriate manner.

Note: Approvals and permissions have been taken in case of reproducing of the dataset.

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Development of Modern Tools for Environmental Monitoring of Pathogens and Toxicant

9

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Abstract

Environmental monitoring is required to protect our surrounding from contamination, especially bacteria, virus, and parasitic pathogens & their toxins as well as chemical substances that can be released into a air, soil, and water create serious public health concerns. Presently, traditional methods more popular for the detection of pathogens and its toxins, but they have several limitations due to low concentrations and interference with various enzymatic inhibitors in the environmental samples. This chapter describes the current state of modern tools, the advantages over conventional detection methods, and the challenges due to testing of environmental samples. Future trends in the development of novel detection devices and their importance, use over other environmental monitoring methodologies are also discussed.

9.1 Introduction

For the past few decades, environmental security has become one of the global challenges. Several emerging pollutants (both biological and chemical) from various sources are distributed over environmental matrices. Globally, the problems caused by biological pollutants especially bacterial, viral, and parasitic pathogens and their toxins are likely to be aggravated and pose serious public health concerns. Conventional culture methods of detecting microorganisms in pollutants are based on the integration of the sample into a suitable enriched medium on which the microorganisms can grow multiply and render visual confirmation. These

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conventional test methods are simple, easily resilient, and usually inexpensive. Mostly, conventional methods are very sensitive and laborious and may require several days. Products that are minimally processed have a naturally short shelf life, which prevents the use of many of these conventional methods. Therefore, extensive research work has been executed to reduce assay time and reduce the amount of manual labor by automating methods through the use of interdisciplinary approaches to detect microorganisms and their toxins. It is against this background the detection and monitoring of environmental pollutants are classified into the following groups: Molecular and various sensor-based methods. These methods of detection can be used to effectively combat environmental components and biological tissues. This chapter presents the monitoring technologies for pathogen agents and their toxins and to discuss the advantages, disadvantages, and various characteristics of those methods. It gives an overview of environmental analytical methodologies reliable for public safety and environmental surveillance.

9.1.1 Pathogen and Its Toxins

A pathogen and its toxins that are biological agents cause disease, disability, or seizure to its host. The term is most often used for describing an infectious agent such as a virus, bacterium, protozoa, prion, fungus, or other microorganisms that disrupt the normal physiology of multicellular animals or plants.

9.1.2 Bacteria

Bacteria are microscopic single-celled organisms that flourish in different type of environments. Most catastrophic diseases such as pneumonia, food-borne illness, blood stream infection (sepsis), and sexually transmitted diseases like gonorrhea are caused by bacteria. Pathogenic bacteria, like *Streptococcus* and *Pseudomonas*, cause globally important diseases, such as pneumonia, and *Shigella*, *Campylobacter*, and *Salmonella* cause food-borne illnesses. Hans Christian Gram, a microbiologist, categorizes bacteria into two groups: (a) Gram-positive and (b) Gram-negative bacteria, and the difference between them is in the cell structure of their wall. Nearly 95% of pathogenic bacteria are Gram-negative bacteria, and the rest are Gram-positive bacteria.

9.1.3 Virus

A virus is a small infectious agent that cannot replicate itself. Viruses have either DNA or RNA as genetic material. It can infect all types of life forms, from animals and plants to microorganisms, including bacteria and archaea and directly instruct the cell machinery to reproduce more and more virus cells.

9.1.4 Fungus

A fungus is a eukaryotic organism that admits microorganisms such unicellular yeast and multicellular molds. These are classified under a separate kingdom "fungus," which includes approximately 100,000 described species. Fungi have both useful and harmful properties. In environmental fungi along with its mycelium, various propagules and the metabolites, it produces mycotoxin.

9.1.5 Significance of the Problem

The monitoring of pathogens and their toxins is one of the core issues in understanding and controlling risk to human health. Problem of pathogen and its toxin contaminants has mild-to-severe and short-term or long-term effects and at some circumstances it causes fetal effect and can become a pandemic. Very strict legislation is implemented in areas such as the environment hazards, in order to prevent the terrible consequences of pathogen and its toxins. Thus, there has been a keen interest in designing and developing tool and techniques for the monitoring of pathogens, that is portable and highly robust assays. From this perspective, different type of monitoring tools has achieved intense significance because of their capability to resolve a potentially large number of problems and challenges in pathogen contamination. This chapter aims to provide new trends in the area of pathogen and its toxins detection.

9.2 Detection of Pathogen and Its Toxins

Environmental pollution adversely affects human health and socioeconomic development. Therefore, it is necessary to develop specific and sensitive monitoring protocols in order to avoid false-positive and false-negative results.

9.2.1 Molecular-Based Detection Methods

During the past decade, the use of molecular methods has supplied the means for examining microbial diversity and detecting specific organisms without the need for cultivation. Several molecular techniques have been developed and extensively used for detecting and typing pathogens. These are evaluated in terms of their performance like discriminatory power, reproducibility, and agreement between typing techniques. The application of molecular techniques to the study of natural and engineered environmental systems has enhanced our insight into the interactions of microorganisms in large and complex environments (Table 9.2). Molecular techniques have also been widely used in surveillance, mutation, and other genetic studies of pathogens to increase our understanding about the primary source of pathogens, source of infection, and genetic diversity. Molecular techniques have

Table 9.1 Advantages and disadvantages of some commonly available molecular techniques for identifying food-borne pathogens

Identification method	Advantages	Disadvantages	Reference
Single PCR	Provides a more accurate, sensitive, and rapid detection of single bacteria or genes	Does not produce isolates that can further be characterized, components in foods can interfere with PCR performance and give misleading results, and PCR conditions must be optimized for better performance	Sails et al. (1998), Wang et al. (2000), Abulreesh et al. (2006)
Multiplex PCRa	Reduces cost, limits sample volumes, and allows rapid detection of multiple bacteria	Primer design is critical, as primers may interfere with each other leaving some genes and bacteria undetected	Elnifro et al. 2000, Shi et al. (2010)
Real-time PCRb	Shortens detection time, detects and quantifies bacteria in real time, and possesses high sensitivity, specificity, and reproducibility	Requires expensive equipment and reagents and setting up requires high technical skills	Heid et al. (1996), Wong and Medrano (2005), Shi et al. (2010)
Reverse transcription PCR b	Can detect only viable cells of pathogens	Much skill is required to handle unstable RNA for pathogen detection	Sails et al. (1998), Sharma (2006), Shi et al. (2010)
Nested PCR	Has improved sensitivity and specificity than the conventional PCR method	Contamination level can be high probably from the laboratory environment	Picken et al. (1997)

Source of Table: Adzitey et al. (2013)

the advantage that they are rapid, less laborious, and more sensitive, specific, and efficient (Table 9.1).

9.2.1.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an in situ DNA replication process that allows for the exponential amplification of target DNA in the presence of synthetic oligonucleotide primers and a thermostable DNA polymerase (Farber 1996; Wang et al. 2000). A wide range of different concentrations or units of DNA templates (5–25 ng), Taq DNA polymerase (0.6–1.25 U), primers (100 μM), and temperature cycles (45–95.8 °C and 30–40 cycles) have been employed to detect or confirm bacteria isolated from environmental pollution (Boonmar et al. 2007; Rahimi et al. 2011; Su et al. 2011). Other components of a PCR reaction such as deoxyribonucleotide triphosphates (dNTPs), magnesium (Mg^{2+}), and buffer solutions have been used in different concentrations to increase detection limits. A PCR process may involve the use of one primer (single PCR) or multiple primers (multiplex PCR) to

Table 9.2 Molecular methods applied to type or characterize bacteria

Typing method	Advantages	Disadvantages	References
PFGE	Has high discriminatory power, reproducibility, and typeability	Requires 3–5 days to complete a test, the cost is relatively high compared to other methods, and this technique has limited availability	Wassenaar and Newell (2000), Trindade et al. (2003)
MLST	Typing data are readily available via the internet, and it is easy to compare results among laboratories and countries, and has good discriminatory ability	This method is expensive and will require skilled researcher to perform	Enright and Spratt (1999), Urwin and Maiden (2003), Dingle et al. (2005)
RAPD	Cheap, rapid, readily available, and easy to perform	Has average reproducibility, discriminatory power, and approximately 80% typeability	Wassenaar and Newell (2000), Shi et al. (2010)
DNA sequencing	Has high discriminatory power, typeability, and reproducibility	Requires more days to complete a test, and this method is complex and relatively expensive	Newell et al. (2000), Wassenaar and Newell (2000)
Denaturing gradient gel electrophoresis (DGGE)			
REP	Cheap, easy to perform, and applicable to small or large number of isolates	Discriminatory power, reproducibility, and typeability are lower compared to PFGE, MLST, and DNA sequencing	Versalovic et al. (1991), Trindade et al. (2003)
ERIC	Quick, cost effective, and does not require much skills to perform	Discriminatory power, reproducibility, and typeability are lower compared to PFGE, MLST, and DNA sequencing	Wassenaar and Newell (2000), Tobes and Ramos (2005)
Ribotyping	Has 100% typeability, good reproducibility, and discriminatory power	It is a complex method and requires 3–4 days to complete a test	Denes et al. (1997), Wassenaar and Newell (2000), Shi et al. (2010)
AFLP	Has good discriminatory power, good reproducibility, and 100% typeability	Requires 3–4 days to complete a test and major capital investment	Wassenaar and Newell (2000), Meudt and Clarke (2007)
RFLP	Inexpensive and very sensitive for strain identification or differentiation	Slow, difficult, and could take up to a month to complete	Mohran et al. (1996), Nachamkin et al. (1996), Babalola (2003)

detect bacterial isolates (Table 9.1). Microorganisms contain a number of well-conserved genes, such as the ribosomal 16S gene and the heat-shock protein/chaperonin Hsp60/65 (or GRAEL), which are excellent targets for PCR. Analysis of the16S ribosomal RNA gene in bacteria in PCR and subsequent sequencing is particularly informative, as there are well-conserved sequences that can be used as binding sites for universal PCR primers adjacent to variable sequences and then a database of known sequences can be compared and analyzed. Other forms of PCR are real-time PCR, nested PCR, reverse-transcription PCR, and many more. Polymerase chain reaction assays have been routinely used for rapid detection, identification, and differentiation of pathogens. They have been used in areas such as DNA cloning, diagnosis of hereditary and infectious diseases, identification of genetic fingerprints, and detection and diagnosis of infectious diseases. Polymerase chain reaction technique plays an important role in the identification of typical bacterial strains that exist in viable but nonculturable coccoid forms (e.g., *Campylobacter* spp.), which are often missed by the conventional method (Magistrado et al. 2001). The use of PCR also avoids situations where phenotypic characteristics are ambiguous and wrongly interpreted, for instance, the occurrence of hippurate negative *C. jejuni* strains (Adzitey and Corry 2011). However, some PCRs may not be suitable for processed and certain foods because amplification can be obtained from DNA originating from both viable and nonviable cells (Sails et al. 1998; Wang et al. 2000). The technique can be expensive and its sensitivity and performance can be inhibited by components of enrichment broth and DNA extraction solution, concentration of the PCR mixtures (primers, DNA templates, dNTPs, and Mg^{2+}), and temperature and cycling conditions (Rossen et al. 1992; Wilson 1997; Wassenaar and Newell 2000). Table 9.1 shows commonly available molecular techniques that have been applied to identify bacteria isolated from environmental samples, while Table 9.2 summarizes the advantages and disadvantages of some commonly available molecular techniques for identifying pathogens. After popularization of polymerase chain reaction (PCR), nucleic acid-based assays for the detection and identification of environmental pathogens have been successfully developed. There are several DNA-based assay formats here, but only nucleic acid amplification techniques have been developed commercially to detect pathogens. PCRs, involving amplification step, are becoming more popular due to their higher sensitivity and fast identification of the pathogens and their toxins. Naravaneni and Jamil (2005) had standardized PCR-based technique for detection of *Salmonella* and *Escherichia coli*. They designed specific genes for examples for *Salmonella* used fimA, pathogenic *E. coli* used afa gene primers for amplification. Adleyb et al.'s (2009) studies have established that BCFomp1/BCRomp1, the DNA sequences, can be used for the specific detection of the *B. cereus* group spp. Analysis of these primers using standard PCR analysis showed that the minimum level of detection was 10^3 CFU/ml and the lowest number of bacterial cell per reaction tube amplified was 5 CFU with initial need of DNA found to be 1 pg. Malorny et al. (2004) developed robust real-time PCR for the specific detection of *Salmonella*. The assay used specifically designed primers and a probe targeted within the *ttrRSBCA* locus, which is located near the *Salmonella* pathogenicity island 2 at centisome 30.5. The

detection probabilities were 70% when a *Salmonella* cell suspension containing 10^3 CFU/ml was used as a template in the PCR (5 CFU per reaction) and 100% when a suspension of 10^4 CFU/ml was used. Sharma (2006) developed a method for detection of mRNA encoded by rfbE and eae genes of enterohemorrhagic *Escherichia coli (EHEC) O157:H7*. A 129-bp and a 106-bp sequence specific to rfbE and eae, respectively, were targeted for real-time detection. This method may contribute to meet the enhancing demand for quality assurance laboratories as standard diagnostic methods. Obeid et al. (2003) characterized and developed reusable glass chip-based microfibricated monolithic microdevices using reverse transcription (RT) and functional integration of PCR in a continuous flow mode. This allows the selection of the number of chip amplification cycles. Samples and reagents for PCR were pumped continuously through appropriate entry holes. After cycles 20, 25, 30, 35, and 40, products were collected from outlet channels. Products were collected in 0.2 ml tubes and analyzed by agarose gel electrophoresis and ethidium bromide staining after 30 cycles in only 6 min. The requirement of the initial DNA and RNA input molecules was used during these studies in the range of $2.5 \times 10^6 - 1.6 \times 10^8$, respectively.

Emerging molecular techniques, such as pulsed field gel electrophoresis (PFGE), denaturing gradient gel electrophoresis (DGGE), multilocus sequence typing (MLST), random amplified polymorphism deoxyribonucleic acid (RAPD), plasmid profile analysis, and deoxyribonucleic acid (DNA) sequencing are among most often used typing techniques and have been applied to pathogens isolated from environmental samples (Table 9.2). Others such as repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), ribotyping, amplified fragment length polymorphism (AFLP), and restriction fragment length polymorphism (RFLP) and so on are yet to be reported in terms of their application. Table 9.1 summarizes the advantages and disadvantages of some commonly available molecular techniques for typing or characterizing pathogens.

9.2.1.2 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is often used to examine microbial diversity of environmental samples and to monitor changes in microbial communities. The number, exact position, and intensity of bands in a gel track in DGGE gel numerically approximate the number and relative abundance of dominant ribotypes in the sample. This approach allows comparison of different microbial communities. Banding patterns of highly diverse microbial communities, present in soils, activated sludge and sediment, are usually very complex when bacterial primer is used. Furthermore, only the major populations of the analyzed community are represented on these DGGE patterns and thus are relatively less abundant but possibly very important species that cannot be detected by this molecular method. The DGGE gel provides a valuable tool for monitoring the structure and dynamics of microbial populations over time or under the influence of environmental changes. This approach has already been used in a few studies, which investigated specific microbial groups such as methanotrophic members of the Proteobacteria, actinomycetes, ammonia-oxidizing bacteria, Archaea, and fungi. Lee et al. (2009)

study Yellow Sand dust, a seasonal meteorological phenomenon affecting East Asia. These storms often provide long-range transport to various microorganisms. Microbiological air samples were collected using a PM2.5 cyclones, Yellow Sand events, and non-Yellow Sand events. Total nucleic acids were also extracted, and the 16S rDNA was amplified by PCR and analyzed by denaturing gradient gel electrophoresis (DGGE). Dendrogram analysis, based on DGGE, indicated that the microbial profiles from the Yellow Sand were distinctive from those of the non-Yellow Sand samples. These results suggest that, as a result of Yellow Sand events, humans in the affected regions are exposed to communities of microorganisms that might cause various adverse health effects. In DGGE, group-specific 16S rRNA primers are useful to compare different microbial communities, as well as to monitor microbial communities in function of time.

9.2.1.3 Pulsed Field Gel Electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) is an agarose gel electrophoresis technique used for separating larger pieces of DNA by applying electrical current that periodically changes direction (three directions) in a gel matrix unlike the conventional gel electrophoresis where the current flows only in one direction (Schwartz and Cantor 1984; Arbeit 1999; Trindade et al. 2003). In PFGE, intact chromosomes are digested using restriction endonucleases to generate a series of DNA fragments of different sizes and patterns specific for a particular species or strain (Shi et al. 2010). This method has good reproducibility, discriminatory power, and typeability, but PFGE is sensitive to genetic instability, has limited availability, and requires at least 3–4 days to complete a test (Wassenaar and Newell 2000).

9.2.1.4 Multilocus Sequence Typing (MLST)

Multilocus sequence typing (MLST) is an unambiguous, portable, and nucleotide-based technique for typing bacteria using the DNA sequences of internal fragments of multiple housekeeping genes (Maiden et al. 1998; Spratt 1999; Urwin and Maiden 2003). In MLST, approximately 450–500 bp internal fragments of each gene are used and most bacteria have enough variation within the house-keeping genes to provide many alleles per locus, thus allowing billions of distinct allelic profiles to be differentiated utilizing the multiple house-keeping loci (Enright and Spratt 1999; Urwin and Maiden 2003). The advantages of MLST are that it provides typing data that are unambiguous, portable, more accurate, and more discriminatory for most bacteria. These data are readily available, comparable, and accessible via the internet in contrast to most typing procedures involving the comparison of DNA fragment sizes on a gel (Dingle et al. 2005). Furthermore, MLST data can be used to investigate evolutionary relationships among bacteria (Urwin and Maiden 2003).

9.2.1.5 Random Amplified Polymorphism Deoxyribonucleic Acid (RAPD)

Random amplified polymorphism deoxyribonucleic acid (RAPD) is a PCR-based technique in which arbitrary primers (typically 10-mer primers) are used to randomly amplify segments of target DNA under low-stringency PCR condition (Wassenaar and Newell 2000). This process leads to the amplification of one or more DNA

sequences and generates a set of finger printing patterns of different sizes specific to each strain (Farber 1996; Trindade et al. 2003). The advantages of RAPD are that it is relatively cheap, rapid, readily available, and easy to perform (Wassenaar and Newell 2000; Shi et al. 2010; Rezk et al. 2012). In RAPD, the efficiency of amplification, annealing, and the length of the product varies with the primed sites, giving rise to both weak and strong amplicons, which makes interpretation of the results difficult. In addition, RAPD has low reproducibility, average discriminatory power, and approximately 80% typeability (Wassenaar and Newell 2000). The use of two or more primers improves the discriminatory power of RAPD (Trindade et al. 2003).

9.2.1.6 Deoxyribonucleic Acid (DNA) Sequencing Techniques

Deoxyribonucleic acid (DNA) sequencing techniques involve technologies used to determine the order of the nucleotide bases (namely adenine, cytosine, guanine, and thymine) in a DNA molecule. In recent times, DNA sequencing is widely and routinely used in the identification, typing, characterization, and/or taxonomic classification of unknown or novel pathogens isolates by many researchers. DNA sequencing has always been preceded by PCR to amplify the target genes. 16S rRNA is a common gene that is amplified for sequencing and subsequently for the identification, typing, and/or taxonomic classification of the pathogen in question. Sequencing has high discriminatory power, 100% typeability, and good reproducibility (Newell et al. 2000; Wassenaar and Newell 2000). The disadvantage is that it requires 2–3 days to complete a test, has limited availability, and costs are higher than other typing methods (Newell et al. 2000; Wassenaar and Newell 2000).

Other typing methods are enterobacterial repetitive intergenic consensus (ERIC), repetitive extragenic palindromic (REP), ribotyping, amplified fragment length polymorphism (AFLP), and restriction fragment length polymorphism (RFLP). Enterobacterial repetitive intergenic consensus (ERIC) PCR uses primers specific for enterobacterial repetitive intergenic consensus sequences. These primers can be used under high stringency conditions to match the target DNA to produce DNA finger printing that are different in sizes (Wassenaar and Newell 2000; Trindade et al. 2003). Enterobacterial repetitive intergenic consensus (ERIC) PCR is quick, easy to perform, and cost effective. Nonetheless, reproducibility is low compared to pulsed field gel electrophoresis. Repetitive extragenic palindromic sequences (REP) also depend on repetitive DNA elements present in pathogens (Trindade et al. 2003). In repetitive extragenic palindromic sequences, repetitive DNA elements present within bacterial genome are amplified to produce finger printing of different sizes specific to each strain (Versalovic et al. 1991). Trindade et al. (2003) reported that REP is cheaper, easy to perform, and applicable to small or large number of isolates, and the results have a good correlation with those obtained by PFGE but have lower discriminatory power. Ribotyping is a molecular technique that uses unique DNA sequences to differentiate strains of bacteria. In ribotyping, first isolation of genomic DNA then digestion of isolated DNA with selected restriction endonuclease at specific sites and generates pieces of DNA of different lengths, then go for separation of pieces of DNA by gel electrophoresis and at last identified bands of DNA using

Southern blot hybridization with specific probe of rRNA genes (Shi et al. 2010). Ribotyping has 100% typeability and good reproducibility, but it is a complex method, is sensitive to genetic instability, and requires 3–4 days to complete a test (Wassenaar and Newell 2000). Ribotyping has higher discriminatory power at the species and subspecies level compared to the strain level (Denes et al. 1997; Shi et al. 2010). Amplified fragment length polymorphism (AFLP) involves the use of two restriction enzymes to digest total genome DNA, one with an average cutting frequency (4-bp recognition site) and the other with a higher cutting frequency (6-bp recognition site) followed by linking of adapters to the sticky ends of the restriction fragments and amplification of a subset of selected restriction fragments (Wassenaar and Newell 2000; Shi et al. 2010). The primers used for amplification are radioactive or fluorescent labeled, and denaturing polyacrylamide gel analysis is used to determine the presence or absence of DNA fragments to identify polymorphisms (Blears et al. 1998; Wassenaar and Newell 2000). Amplified restriction length polymorphism has good discriminatory power, good reproducibility, and 100% typeability, needs no prior sequence information for amplification, and is insensitive to genetic instability, but AFLP is a complex method, requires 3–4 days to complete a test, and requires major capital investment (Wassenaar and Newell 2000; Meudt and Clarke 2007). Restriction fragment length polymorphism (RFLP) involves the use of restriction enzyme to digest DNA and to separate the resulting restriction fragments according to their length on agarose gel electrophoresis. Restriction fragments are then transferred into a membrane through Southern blot procedure and hybridized to a membrane bound labeled DNA probe (Babalola 2003; Foley et al. 2009). This method utilizes the variations in homologous DNA sequences to characterize bacteria. This technique is inexpensive, is very sensitive for strain identification or differentiation, and has widespread application, although it has become obsolete in the present time due to the emergence of relatively inexpensive sequencing technologies (Mohran et al. 1996; Babalola 2003). The technology is also slow and difficult and could take up to a month to complete (Mohran et al. 1996; Nachamkin et al. 1996).

9.2.2 Enzyme-Linked Immunsorbent Assay (ELISA)

Many immunological techniques provide quantitative assessment of the concentration of analytes in pure solutions or complex mixtures. In this area have great potential due to its sensitive and specificity towards diverse range of chemical and biological molecules and the immunoassays can also be used to provide real-time information.

The enzyme-linked immunosorbent assay (ELISA) is a test that identifies the substrate by the interaction of antibodies with antigen. An antibody is “Y”-shaped immunoglobin (Ig) that is made up of two heavy chains (H) and two light chains (L). Each of the chain has a constant and a variable part. The variable part is specific to the antigen that binds with corresponding antigen that is highly specific and selective (Conroy et al. 2009; Donahue and Albitar 2010). An ELISA required at least one

antibody with specificity towards particular antigen. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate. The sample with an unknown amount of antigen is immobilized on a solid support known as a polystyrene microtiter plate either nonspecifically or specifically. After the antigen is immobilized, the primary antibody (detection antibodies) is added, forming a complex with the antigen. The primary antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. The main enzymes used are horseradish peroxidase, alkaline phosphatase, and β-galactosidase. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are nonspecifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

9.2.2.1 Basic Steps for Developing and Running an Immunoassay (Karen et al. 2012)

1. Establish assay critical success factors (i.e., sensitivity required).
2. Ensure that appropriate antibody and antigen reagents are available.
3. Adsorb antigen or capture antibody to a solid surface.
4. Wash off unbound reagents.
5. Block nonspecific binding sites to reduce background.
6. Incubate the secondary antibody with the sample.
7. Wash off unbound reagents.
8. Incubate secondary antibody conjugate with sample.
9. Wash off unbound reagents.
10. Incubate substrate to generate signal.
11. Calibrate curve fitting, data analysis, and quantitation by nonlinear regression.

Enzyme-linked immunosorbent assay (ELISA) is a widely used immunoassay for environmental purposes. Three different types of ELISA are carried out according to different formats: direct competitive, indirect competitive, or sandwich type. Competitive assays are most common and can be performed in different ways. Analyte and the tracer (direct competitive ELISA) or analyte and the immobilized ligand (indirect ELISA) may compete for a limited number of binding sites. Sandwich-type ELISA is a noncompetitive assay, in which the analyte is recognized by two different antibodies, immobilized Ab and marker Ab (Harris 1999; Farre et al. 2005). Flow-injection immunoassay (FIIA) is a technique, based on the introduction of the sample into carrier stream, which enters the reaction chamber where the immunoreaction takes place. FIIA has been successfully used for detection of different pollutants, e.g., triazines. At present, this method is integrated into different immunosensors. Immunoassay and other immune techniques are the powerful and elegant techniques for rapid detection of environmental pathogen and its toxins. They also provide accurate and convenient means of detection of adulteration and authentic assay. These assays are fast and relatively inexpensive. Immunoassays are not as susceptible to matrix effects as PCR assays. Meng and Doyle (2002) and Taitt et al. (2004)

developed single-analyte sandwich immunoassays for the detection of *Salmonella enterica* serovar Typhimurium, with a detection limit of 10^4 CFU/ml; the limit of detection was improved ten-fold by lengthening the assay protocol to 1 h. *S. enterica* serovar Typhimurium was also detected in the following spiked foodstuffs, with minimal sample preparation: sausage, cantaloupe, whole liquid egg, alfalfa sprouts, and chicken carcass rinse. To determine its efficacy as a screening tool for the diagnosis of asymptomatic *Salmonella* infection of poultry, chicken samples were tested and the limit of detection of pathogen was 10^3 CFU/g. The most commonly used immunoassays for the detection of the pathogen are based on the use of whole cells, or heat killed (Silbernagel et al. 2005), or formalin fixed (Solve et al. 2000), and then detected by an ELISA. Feldstine et al. (1997) developed an immunoprecipitation method that made use of heat killed *L. monocytogenes* cells to detect contamination between 0.003 and 11 cfu/ml of food samples. An enzyme-linked immunosorbent assay (ELISA) for *Clostridium botulinum* type A and type B toxins was assessed for diagnostic accuracy in cases of infant botulism. Botulism is a serious food-borne neuroparalytic disease, caused by botulinum neurotoxin (BoNT), produced by the anaerobic bacterium *Clostridium botulinum*. Stanker et al. (2013) developed serotype B-specific monoclonal antibodies for sandwich (capture) ELISA antibodies ranging from 10 to 48×10^{-11} M. Assay performance for all possible combinations of capture–detector antibody pairs was evaluated, and the antibody pair resulting in the lowest level of detection (L.O.D.) ~20 pg/mL was determined.

9.2.3 Laser-Induced Breakdown Spectroscopy

Laser-induced breakdown spectroscopy (LIBS) has a flexible and convenient technique for rapidly determining the elemental composition of samples with minimal or no sample preparation. This technique is used to analyze the spectral emission from laser-induced plasmas, the plasma emission intensity being proportional to the abundance of an element in the sample. The relative simplicity and capability of fast multielemental analyses of solid, liquid, or gaseous samples make LIBS an ideal tool to study a wide range of samples. Although the use of LIBS has been most popular in metallurgical and biological samples, in recent years, it has been used to study environmental and biological samples. Yu et al. (2010) separated a variety of bacteria by detecting the trace mineral elements contained in five different types of bacteria that were grown in the same nutrient liquid, among them are four Gram-negative species (*Acinetobacter baylyi*, *Erwinia chrysanthemi*, *Escherichia coli*, and *Shewanella oneidensis*) and one Gram-positive bacterium (*Bacillus subtilis*). In the next round of this work, they evaluated the performance of LIBS for both sensitive detection of mineral trace elements in fresh vegetables and highly spatially resolved measurements of the amounts. Rosalie et al. (2010) described the use of LIBS to differentiate live pathogens and killed viruses on substrates. They differentiat the live pathogens *B. anthracis* Stern strain and *F. tularensis* live vaccine strain colonies on agar and demonstrate that it was found possible to differentiate between a

samples. UV-killed *hantavirus* strains were studied as dilutions on slides, and it was also found possible to differentiate between strains. Jonathan and Pourmand (2007) also identified and compared a pathogenic with nonpathogenic strain by LIBS. In their experiment they identified a pathogenic strain of bacteria, *Escherichia coli* O157:H7 enterohemorrhagic *E. coli* or EHEC, and compared three nonpathogenic *E. coli* strains (a laboratory strain of K-12 AB), a derivative of the same strain termed HF4714, by LIBS with nanosecond pulses in environmental samples. Multari et al. (2012) described the rapid detection of biological contaminants, such as *Escherichia coli O157:H7* and *Salmonella enterica*, on perishable foods items present in environment. Here, multivariate regression analysis of LIBS data is used to differentiate the live bacterial pathogens *E. coli O157:H7* and *S. enterica* in various foods (eggshell, milk, bologna, ground beef, chicken, and lettuce) and surfaces (metal drain strainer and cutting board). Qassem et al. (2011) investigated the effect that adverse environmental and metabolic stresses have on the laser-induced breakdown spectroscopy (LIBS) identification of bacterial specimens. Single-pulse LIBS spectra were acquired from a nonpathogenic strain of *Escherichia coli* cultured in two different nutrient media: a trypticase soy agar and a MacConkey agar with a 0.01% concentration of deoxycholate. A chemometric discriminant function analysis showed that the LIBS spectra acquired from bacteria grown in these two media were indistinguishable and easily discriminated from spectra acquired from two other nonpathogenic *E. coli* strains. Samuels et al. (2003) also used laser-induced breakdown spectroscopy to study bacterial spores, molds, pollens, and proteins. Biosamples were prepared and deposited onto porous silver substrates. LIBS data from the individual laser shots were analyzed by principal components analysis and were found to contain adequate information to afford discrimination among the different biomaterials.

9.2.4 Need of Sensors

Environmental pollution in various media is a serious health concern worldwide. Hence there is a continuing need to develop a cost-effective, accurate, fast, reliable, noninvasive, and nondestructive methods or tools for fast, analytical techniques used in comprehensive monitoring programs. Humans have sensors to understand and detect the environment around them. Therefore, it is equally important to design and develop biosensor-based measurement techniques that can accurately detect various contaminants from a wide spectrum. However, biosensors have several limitations for environmental analysis including (1) response time, (2) sensitivity, (3) selectivity, (4) compatibility, (5) affinity, (6) stability, (7) lifetime, etc.

9.2.4.1 Biological Sensor

A biosensor is a device that can be used to convert the existence of a molecule or compound into a measurable and useful signal. Biosensors use excitation to translate changes into recognizable signals. In 1962 Clark and Lyons developed a fast and more precise biosensor for glucose measurement. Biological sensors are analytical

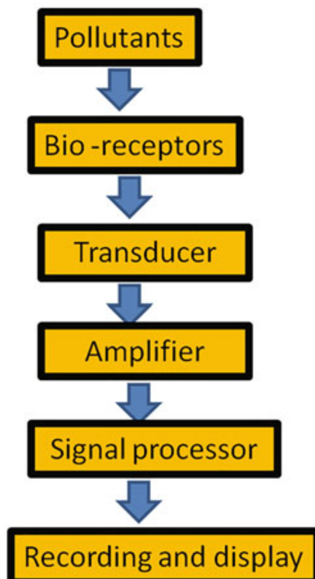


Fig. 9.1 Biological sensor (source of picture: modification of Costa et al. 2012)

devices that detect biochemical and physiological changes. A biosensor is defined by the International Union of Pure and Applied Chemistry (IUPAC) as a self-contained integrated device that is capable of providing specific quantitative or semiquantitative analytical information using a biological recognition element (biochemical receptor), which is retained in direct spatial contact with a transduction element. Transducers are essential to convert the particular biological and chemical changes into electrical data, which can identify different biochemical components of a complex compound to isolate the desired biochemical compounds (Fig. 9.1).

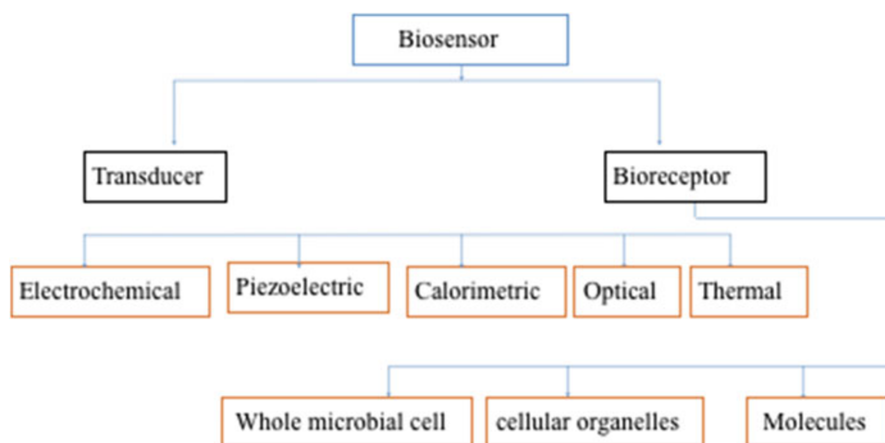
Basically, biosensors can be divided into two broad classes: (1) based on the signal transduction they employ and (2) based on the biological signaling mechanism they utilize. In the transducing element, class biosensors can be characterized as electrochemical, bioluminescence, optical, piezoelectric, and thermal sensors. A wide range of biological recognition elements have been used in biosensors constructed for potential environmental applications. Whole microbial cells, cellular organelles, and molecules such as enzymes, antibodies, different kinds of receptors, or DNA are the most common biorecognition elements of microbial origin.

Based on Transduction

Electrochemical Biosensors: Electrochemical biosensors measure the current produced from oxidation and reduction of an electroactive species in a biochemical reaction. These are generally based on biocatalysis of a reaction that produces or consumes electrons (such enzymes are rightly called redox enzymes). The sensor substrate usually contains an electrode that is used as the transduction element. These biosensors have the ability to even operate in turbid media. Electrochemical biosensors emerge as the most commonly used biosensors in monitoring and diagnosis of samples in environmental and clinical analysis (Vargas et al. 2018).

Optical biosensors are more suitable for direct monitoring systems. An optical biosensor is a compact analytical tool containing a biorecognition sensing element integrated with an optical transducer system. The basic objective of an optical biosensor is to produce a signal that is proportionate to the concentration of a measured substance (analyte) (Damborsky et al. 2016). The optical biosensor can use various biological materials, including enzymes, antibodies, antigens, receptors, nucleic acids, whole cells, and tissues as biorecognition elements. Optical-based biosensor is able to provide a direct, real-time, and label-free detection of many biological and chemical substances (Fan et al. 2008, Bhatta et al. 2012). The surface plasmon resonance (detailed in Sect. 9.2.4.2) or fluorescence that is integrated with optical fiber is a most popular method available for optical-based biosensing (Caygill et al. 2010).

Piezoelectric biosensors are developed by coating the surface of the biosensor with a selectively binding biologically active substance (Maraldo and Mutharasan 2007). Piezoelectricity can be explained as a linear interaction between mechanical and electrical systems in noncentric crystal or similar structure, which was first discovered by Curie brothers in 1880 (Pohanka 2018). Piezoelectric biosensors are a group of analytical devices working on the principle of affinity interaction. In piezoelectric biosensor, the transducer is made of piezoelectric material (e.g., quartz) and the biosensing material that coated on the piezoelectric material which vibrate at the natural frequency.



Based on Bioreceptor

Microbial Biosensors: A microbial biosensor consists of a transducer in conjunction with immobilized viable or nonviable microbial cells. They may be categorized into one of the two groups. The first group of biosensors is viable organisms, targeted to measure an integral toxicity, genotoxicity, estrogenicity or other general parameters of the sample. They essentially include whole microorganisms as biorecognition

elements. The most often reported cell-based biosensors include genetically modified bacteria with artificially constructed fusions of particular regulatory system (native promoter) with reporter genes. The presence of an effector (nonspecific such as DNA damaging agents, heat shock, oxidative stress, toxic metals, organic environmental pollutants) results in transcription and translation of fused target genes, generating recombinant proteins that produce some measurable response. Frequently used reporter genes are lux (coding for luciferase) and gfp (coding for green fluorescence protein), expression of which correlates with luminescence- or fluorescence-based light emission. Colorimetric determination of target gene expression is possible by fusing it to reporter genes coding for β-galalactosidase (lacZ) or alkaline phosphatase (phoA). Recently, *E. coli* biosensor capable of detecting both genotoxic and oxidative damage has been developed by introducing plasmids with fusion of katG (gene encoding for an important antioxidative enzyme) promoter to the lux reporter genes, and another with recA (gene encoding crucial enzyme for DNA repair) promoter with the gfp reporter gene (Mitchell and Gu 2004). Microbial biosensors have been widely used in the environmental, food, and diagnostics industry due to its advantages of low cost, stability, and fast response. Compared to enzymes, the microorganisms that are used as bioelements can make use of the enzyme to specifically respond to the analytes without time-consuming and expensive purification. Based on its attractive properties, several directions for the development of the microbial biosensors have shown great promise.

DNA/Nucleic Acid Sensor: Genetic information can be used as a biorecognition part of various biosensors. Identification of pathogen from a human tissue or blood samples are common analytes for these biosensors. This biosensor principal is based on recognition of the complementary strand by ssDNA to form stable hydrogen bond between two nucleic acids to become dsDNA. In order to achieve this, ssDNA is used as probe to immobilized in bioreceptor and complementary sequences present in the target of interest. The highly specific affinity binding's reaction between target to the probe's single strand DNA, which results in hybridization of complementary ssDNA to form dsDNA. Subsequently biochemical reaction that allows transducer amplified the signal into electrical one. Sometimes linker such as thiol or biotin is needed in the effort to immobilize the ssDNA onto the sensing surface. The nucleic acid biological recognition layer, which incorporates with transducer, is easily synthesizable, highly specific, and reusable after thermal melting of the DNA duplex (Fig. 9.2). Moreover, Yeh et al. (2011) have reported optical biochip for bacteria detection based on DNA hybridization with detection limit of 8.25 ng/ml. However, electrochemical transduction is the most abandoned method used to study DNA damage and interaction, as reported in the literature. The development of electrochemical DNA biosensor has received a great deal of attention lately, and this has largely been driven by the need to develop rapid response, high sensitivity, good selectivity, and experimental convenience (Liu et al. 2012).

9.2.4.2 Surface Plasmon Resonance (SPR)

The phenomenon of surface plasmon resonance biosensor was first reported by Wood (1902). The application of biomolecule interaction was first reported by

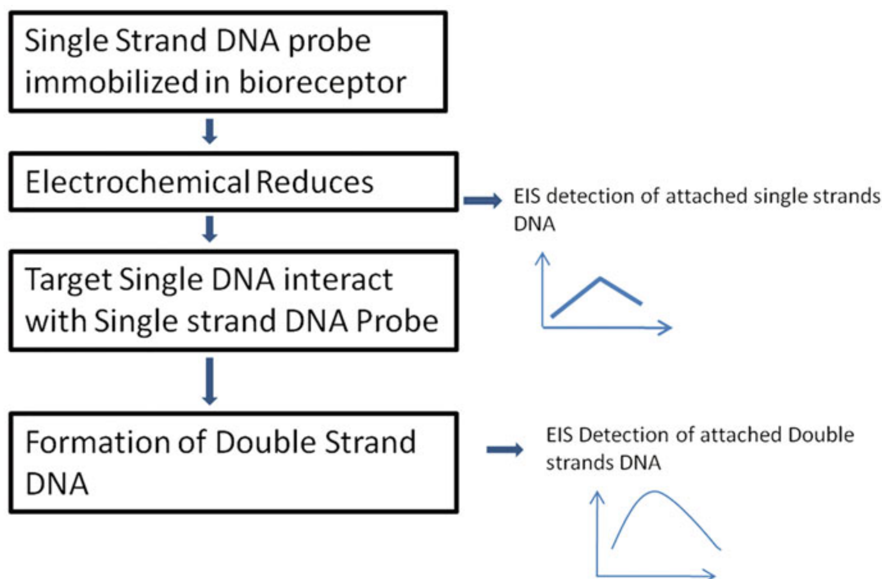


Fig. 9.2 Schematic diagram of the fabrication of the impedimetric DNA biosensor and the detection of target DNA (source: modification of Q. Gong et al. 2015)

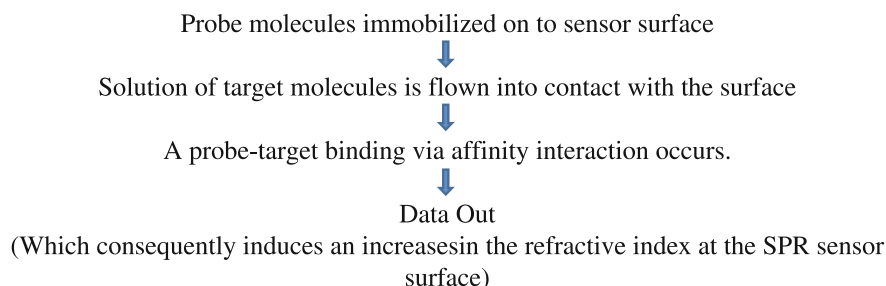


Fig. 9.3 Flow diagram show surface plasmon resonance system

Liedberg et al. 1983, and the complete phenomena of excitation of surface plasmon were explained by Otto (1968). Surface plasmon resonance biosensor (SPR), a modern, cutting edge sensor technology, can perform rapid detection of pathogen (Fig. 9.3). SPR is the optical sensor that provides sensitive, label-free, and real time (few seconds or minutes) monitoring of reaction and has been proven to be one of the most powerful technologies to determine specificity, affinity, and kinetic parameter during the binding of macromolecules in many bond types including protein–protein, DNA–protein, lipid–protein, polysaccharides–protein, and virus protein, among others. Identification of biomolecules on SPR was made possible by immobilizing a capturing agent, such as antibodies, enzyme, peptide, and DNA on

a metal surface, allowing the sample solution flow in excess over that surface, while using SPR spectroscopy to measure the changes in the SPR angle, which is the angle of minimum reflectivity, it can be determined by varying the incidence angle and recording the reflected light intensity during the biological binding reaction between various biomolecules. So far, numerous studies have advanced the potential of SPR sensors by increasing the effectiveness of the techniques.

SPR system offers a simple means of identifying bacteria, even a very small number of bacteria in real time, without any markers. The bacteria interact with specific ligands grafted on the chip, to bring about a local change in the refractive index in the vicinity of surface and then a plasmonic resonance signal. Using SPR system in imaging allows numerous different probes to be attached to the chip's surface measuring, so that numerous pathogens can be simultaneously identified in the course of a single test. SPR-based biosensors have been reported by many researchers for the detection of food-borne pathogens such as *L. monocytogenes* (Koubova et al. 2001), *Salmonella* (Koubova et al. 2001; Oh et al. 2004), and *E. coli O157:H7* (Subramanian et al. 2006; Waswa et al. 2007). Also, commercially available optical biosensors use SPR for monitoring and identifying pathogens and their toxins especially in environmental pollution.

9.2.4.3 Carbon Dioxide Sensor

Carbon dioxide sensor is a device for the measurement of elevated CO_2 gas level from biomedical studies to food-packaging processes. As the role of these gases, in the determination of air quality by biochemical reactions, Now a days, development of different types of CO2 sensors such as optical sensors, polymer opal films, polymer hydrogels, etc., by using different fabrics, such as solid electrolyte, mixed oxide capacitors, polymers with carbonate solution and so on, have been investigated (Lai et al. 2011). Among them, solid electrolyte-type CO_2 sensors are of particular interest from the viewpoint of low-cost, high-sensitivity, high-selectivity, and simple element structure (Santonico et al. 2017). There are needs of efficient CO_2 sensors that can intelligently monitor the gas concentration changes. Hence, a CO_2 sensor incorporated into package can efficiently monitor product quality. Although much progress has been made so far in the development of sensors monitoring CO_2, most of them are not versatile and suffer from limitations such as high equipment cost, bulkiness, and energy input requirement, including safety concerns. Latest approaches, for more compatible with industrial demand, would consist of printable sensor membranes on the packaging material and should provide information about analytes at any given stage in the packaging and delivery process, to sense the physical and biological (microbiological) changes (Mheen and Kwon 1984). Chu and Syu (2017) design a carbon dioxide based an optical sensor for the sensing films coated on filter paper. Ethyl cellulose (EC) doped with platinum (II) meso-tetrakis (pentafluorophenyl) porphyrin (PtTFPP) and 7-amino-4-trifluoromethyl coumarin serve as the oxygen-sensing material and reference blue emission dye for the pH indicator, respectively. The sensing layer includes the pH-sensitive fluorescent indicator 1-hydroxy-3,6,8-pyrenetrisulfonic acid trisodium salt immobilized within the ethyl cellulose. The carbon di oxide sensitive materials

can both be excited with a 405 nm LED, and the two emission wavelengths can be detected separately. The proposed optical dual sensor can be used for the simultaneous sensing of carbon di oxide concentrations in environmental applications.

9.2.4.4 Immunosensor

An antibody-based biosensor was applied for the first time to detection in the 1950s, opening the doors to the possibility of immunodiagnosis (Donahue and Albitar 2010). Since then, there have been vigorous efforts made to develop immunosensor that is composed of antigen/antibody as bioreceptor as a tool for clinical diagnostics (Conroy et al. 2009; Orazio 2011). Hence, an immunosensor is highly specific, stable, and versatile. The specificity of an antibody toward the binding side of its antigen is a function of its amino acids (Fowler et al. 2008). Those days, there are two types of detection methods, which are frequently used in immunosensor, that is, optical and electrochemical. However optical detection transduction method has suffered from poor sensitivity when coupled with radioimmunoassay, the short half-life of radioactive agents, concerns of health hazards, and disposal problems. Electrochemical detection overcomes problems associated with other modes of detection of immunoassays and immunosensors (Fig. 9.4). In contrast, electrochemical immunoassays and immunosensors enable fast, simple, and economical detection, which are free of these problems Fowler et al. (2008). However, recent advance in science and technology has created an optical transduction method, a new path toward highly sophisticated automated instrument. Hence, optical and electrochemical detection methods are gaining mutual importance for development of

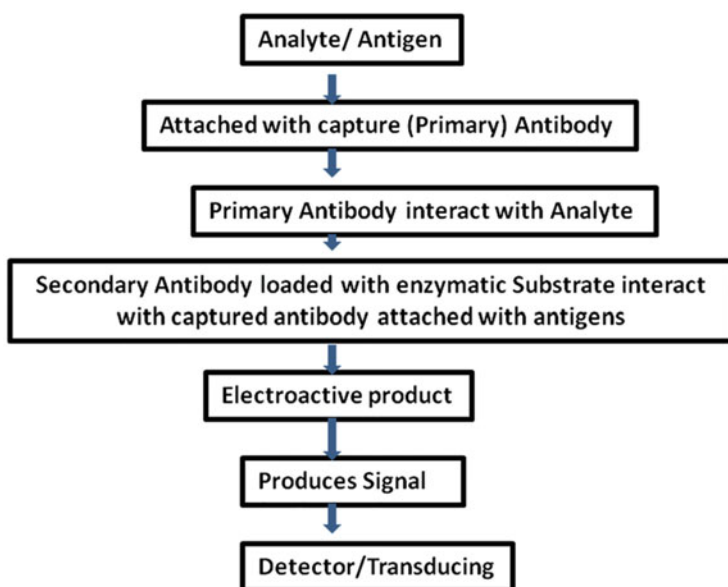


Fig. 9.4 Process of immunosensor (source of picture: modified from Cho et al. 2018)

immunosensor (Shankaran et al. 2007; Bhatta et al. 2012). The development of immunosensor for bacteria and pathogen detection has gained a great deal of attention due to its application in the point-of-care measurement (POC) (Braiek et al. 2012; Holford et al. 2012).

9.2.4.5 Nanosensor

Nanosensor is defined as a modified version of a biosensor or chemical sensor employing nanomaterial in an analytical device, helping in detection of toxic contaminants in environmental toxicant at very sensitive levels. Nanomaterial is defined as material with at least one dimension smaller than 100 nm. Owing to their incredibly small size, nanomaterials can be very versatile in many terms of their detection and monitoring. Nanoparticle-based biosensors are considered as potential tools for rapid, specific, and highly sensitive detection of the analyte of interest (Table 9.3). Nanomaterials can provide optical, catalytic, magnetic, and antimicrobial properties for sensing applications. Therefore, the integration nanotechnology in sensing platforms has provided significant enhancements in detection capabilities and functionality of these devices. However, multifunctional nanosystems have the potential to act simultaneously as a method for rapid microbial capture, detection, and decontamination. Thus, future developments are also expected in the

Table 9.3 Detection of biological contaminant by Nanobiosensor

Nanomaterials	Biological contaminants	Electrode/sensor	Reference
Functionalized AuNPs	Aflatoxin	Immunoelectrode	Sharma et al. (2010)
CNTs	Salmonella	Electrochemical biosensor	Jain et al. (2012)
Glyconanoparticles	Cholera toxin	Colorimetric bioassay	Schofield et al. (2007)
Silver core and a gold shell (AgAu)	AF B1	Immunodipstick assay	Liao and Li (2010)
Nanostructured zinc oxide	Mycotoxin	ITO glass plate	Ansari et al. (2010)
Magnetic nanoparticles and TiO_2 nanocrystals	Salmonella	Optical nanocrystal probes	Joo et al. (2007)
Oligonucleotide-functionalized Au nanoparticles	Escherichia coli O157:H7	Piezoelectric biosensor	Chen et al. (2008)
Liposomic and poly (3,4-ethylenedioxythiophene)-coated CNTs	Cholera toxin	Electrochemical immunosensor	Viswanathan et al. (2006)
Fe_3O_4 NPs	*Campylobacter jejuni*	Glassy carbon electrode	Huang et al. (2010)
AuNPs	Melamine	Colorimetric aptasensor	Yun et al. (2014)

Source: Kuswandi et al. (2017)

development of smart labels to indicate food spoilage or presence of harmful toxins. Thus, this area would benefit from fundamental advances in the development of low-cost and flexible nanosensors suitable for roll-to-roll manufacturing in large-scale production. The use of inexpensive materials such as paper or plastic and integration of all sensing reagents into a portable compact unit is also desirable for future deployment and rapid implementation of these devices. Method validation, comparability, stability, and interlaboratory studies to evaluate performance are also needed to ensure robustness and accuracy of these devices for real-world applications (Mustafa et al. 2017).

9.3 Conclusion

In this chapter, we summarized the recent progress in modern tools for monitoring of environmental pollution and assessment to promote for betterment of the public health and individual life quality. So our center of interest to detect of pathogens in the actual environmental samples is imperative. Design and development of detection methods with sensitivity, reproducibility, selectivity, and speediness are urgently required for screening their occurrence in correspondence with safety regulations at significant levels. The nucleic acid-based biosensors have potential to sense the samples (pollutant) in a very low concentrations, and it is time-effective upstream processes. Immunosensors have relatively fewer steps and required less assay time but needs specific antibodies that are complicated and non-economical. Using different signal amplification and background-reduction techniques coupled with the miniaturization with enhanced sensitivity, nucleic acid/antibody-based detection methods offer sensitive and selective tools for screening various forms of pathogens. Use of nanoparticles and nanomaterials will facilitate efficient techniques, multiplex detection systems, and nanomaterial-based research for simultaneously sensing relevant pathogens in a specific environmental scenario. It has been revolutionized the case of biological detection. The overall mechanism has become robust, smarter, less costly, and user friendly. The significant advantage includes rapid results because the approach to increase signal rather than the target analytes has revolutionized the paradigm of detection.

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10 Interactions of Environmental Risk Factors and Genetic Variations: Association with Susceptibility to Cancer

Munindra Ruwali and Rahul Shukla

Abstract

The biological mechanisms of cancer development involve both environmental and genetic factors. Among the environmental factors, tobacco and alcohol have been established as one of the most potent causative factors in promoting carcinogenesis while genetic variations in phase I and phase II xenobiotic metabolizing enzymes (XMEs) play an important role in determining the outcome of exposure to environmental carcinogens. The genes that contribute to cancer development fall into three broad categories, namely tumor suppressor genes, oncogenes, and DNA repair genes though several other genes are also involved. Cytochrome P450s (CYPs) are the most important super family of phase I XMEs while phase II Glutathione S-transferases (GSTs) conjugate a wide range of electrophilic substrates with the abundant cellular nucleophile-glutathione (GSH) promoting their metabolism, detoxification, and excretion. There has been a lot of interest in unravelling gene–environment interactions, i.e. whether the risk of cancer associated with a particular environmental exposure such as tobacco and alcohol differs with respect to functionally different polymorphisms of these genes. A large number of studies have been conducted involving several genes and many cancers and the results, though sometimes inconsistent, help in advancing the etiological understanding of cancer. Study of gene–environment interaction is important for improving accuracy and precision in the assessment of both genetic and environmental influences in promoting cancer. Gene–environment interaction also has important implications for public

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health as it helps in predicting cancer rates and provides a basis for appropriate recommendations for cancer prevention.

10.1 Introduction

Cancer is caused by changes in genes that alter the way our cells perform their normal functions. Some of these genetic changes occur naturally when DNA is replicated during the process of cell division while others are the result of environmental exposures that damage DNA. Studies have shown that among the factors responsible for cancer, environmental factors play an important role particularly tobacco and alcohol (Brennan and Boffetta 2004). The relationship between cancer development and use of tobacco and alcohol was analyzed by two systematic reviews published by The World Cancer Research Fund in 2007 (World Cancer Research Fund 2007) and the International Agency for Research on Cancer in 2012 (International Agency for Research on Cancer 2012). The reviews concluded that long-term alcohol and tobacco consumption increases risk of cancer of the lips, oral cavity, pharynx, larynx, lung, stomach, colorectum, breast, pancreas, and liver. Similarly, studies have also been conducted that show that there is a relationship between cancer onset and alcohol consumption and tobacco smoking. The higher the amount of alcohol and tobacco consumed, the greater is the risk of cancer in the long term. However, inter-individual variations in susceptibility to cancer have also been observed. Individuals having the same amount and duration of usage of tobacco and alcohol may have differences in susceptibility to cancer which might be explained by gene–environment interactions. The genetic make-up of an individual decides the outcome of an exposure to a carcinogen (Fig. 10.1). Study of gene–environment interaction helps to improve accuracy and precision in the assessment of both genetic and environmental influences. Following sections discuss the risk factors for cancer, namely alcohol and tobacco, genetic variations in key enzymes and gene–environment interactions.

10.2 Environmental Risk Factors and Cancer Risk

10.2.1 Tobacco and Cancer Risk

The use of tobacco is a leading cause of cancer and also death from cancer. People who consume tobacco products or who are regularly around environmental tobacco smoke (also called second hand smoke) have an increased risk of cancer because tobacco products and second hand smoke have components that damage DNA. Processed tobacco leaves possess more than 3000 chemicals (Roberts 1988) which include leaf constituents as well as constituents derived from multiple sources. Unburned tobacco products contain carcinogenic nitrosamines, polycyclic aromatic hydrocarbons (PAHs), radioactive elements, and cadmium. Several forms of tobacco

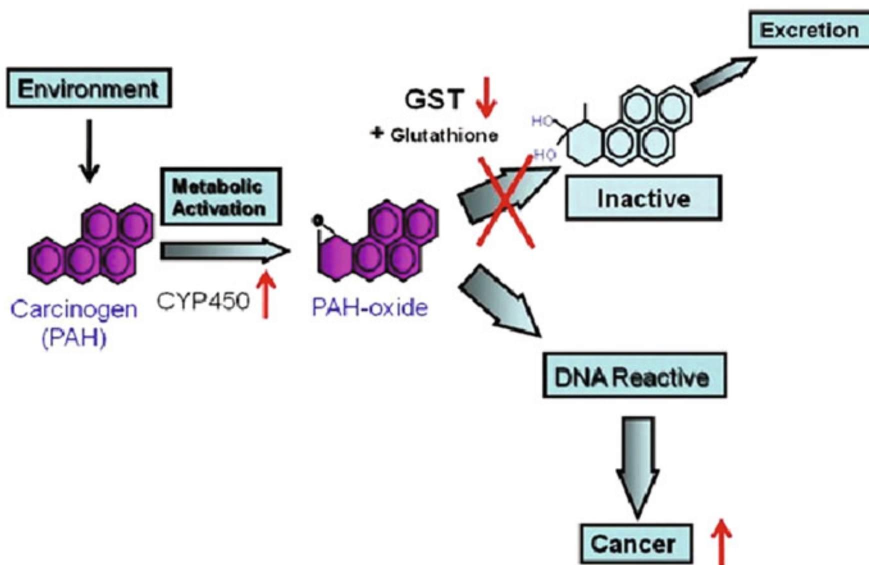

Fig. 10.1 Environmental carcinogens get activated by CYPs and either get detoxified by GSTs followed by excretion from body or cause DNA damage resulting in cancer. However, more expression/activity of CYPs and/or less expression/activity of GSTs will promote carcinogenesis for the same environmental exposure as a result of gene–environment interaction

commonly used are cigarettes, smokeless tobacco, cigars, and pipes (Iribarren et al. 1999). The quality of smoked cigarette and quitting smoking also have a role in oral cancer risk as shown in studies that filtered cigarettes and smoking cessation substantially lower risk of oral cancer (Kabat et al. 1994). The smoke emanating from cigarettes has thousands of chemicals with around 60 of them carcinogens (Table 10.1 lists some of the major components in the cigarette smoke). Cigarette smokers have a lifetime increased risk for head and neck cancers (HNSCC) that are 5–25 fold increased over the general population (Andre et al. 1995). Those who quit smoking are at the same level of risk as of a non-smoker 20 years after smoking cessation. Risks of lung cancer and cancers of the upper respiratory tract increase with increased consumption and duration of smoking. Incidences of laryngeal cancer are strongly connected with exposure to tobacco smoke containing dozens of carcinogens. Genotoxic agents such as PAHs present in tobacco smoke are responsible for lesions of structure of DNA and formation of DNA adducts by metabolically activated intermediates.

Smokeless tobacco products like chewing tobacco and sucked tobacco are the other tobacco related risk factors involved in cancer development (Accort et al. 2005). Simultaneous consumption of betel nuts with smoking and drinking makes one susceptible for esophageal cancer several hundred times. A form of tobacco called “khaini” (mixture of tobacco, lime, and menthol or aromatic species) has very high level of nitrosamines (Stepanov et al. 2005) which can be attributed to the

Table 10.1 Some of the IARC compounds reported in cigarette mainstream smoke

Class	Compound name	Molecular formula
Hydrocarbons		
Aliphatic	Isoprene	C_5H_8
	4-Vinylcyclohexene	C_8H_{12}
Polycyclic	Benzo[*a*]pyrene	$C_{20}H_{12}$
	Benzo[*b*]fluoranthene	$C_{20}H_{12}$
	Benzo[*j*]fluoranthene	$C_{20}H_{12}$
	Benzo[*k*]fluoranthene	$C_{20}H_{12}$
	5-Methyl-chrysene	$C_{19}H_{14}$
	Dibenzo[*a,e*]pyrene	$C_{24}H_{14}$
	Dibenzo[*a,h*]pyrene	$C_{24}H_{14}$
	Dibenzo[*a,i*]pyrene	$C_{24}H_{14}$
	Dibenzo[*a,l*]pyrene	$C_{24}H_{14}$
	Indeno [1, 2, 3-cd] pyrene	$C_{22}H_{12}$
Aromatic	Ethyl benzene	C_8H_{10}
	Styrene	C_8H_8
Nitrogen containing components		
Aromatic amines	*o*-Anisidine	C_7H_9NO
	p-Chloroaniline	$C_6H_6C_lN$
	2,6-Xylidine (2,6-dimethylaniline)	$C_8H_{11}N$
Aza-arenes	Dibenz[*a,h*]acridine	$C_{21}H_{13}N$
	Dibenz [*a,j*]acridine	$C_{21}H_{13}N$
	Dibenzo[*c,g*]carbazole	$C_{20}H_{13}N$
Nitrosamines	*N*-Nitrosodi-n-butylamine (NDBA)	$C_8H_{18}N_2O$
	N-Nitrosodi-n-propylamine (NDPA)	$C_6H_{14}N_2O$
	N-Nitrosoethylmethylamine (NEMA, MEN)	$C_3H_8N_2O$
	N-Nitrosodiethanolamine (NDELA)	$C_4H_{10}N_2O_3$
	4-(*N*-Nitrosomethylamino)-1-(3-pyridinyl) -1-butanone (NNK)	$C_{10}H_{13}N_3O_2$
	N′-Nitrosonornicotine (NNN)	$C_9H_{11}N_3O$
	N-Nitrosopiperidine (NPIP, NPP)	$C_5H_{10}N_2O$
	N-Nitrosopyrrolidine (NPYR, NPY)	$C_4H_8N_2O$

method of tobacco processing which converts nitrate to nitrite (Hoffmann et al. 1995). The consumption of khaini directly affects the oral mucosa via interactions with high amounts of nitrite as well as N-nitroso compounds formed endogenously. This has been seen in saliva of individuals consuming khaini (Stitch et al. 1992). Other forms of popular smokeless tobacco products are Zarda and Gutka. Zarda is produced by boiling tobacco leaves in water with lime and spices and has significantly high levels of nitrosamines, while Gutka contains less nitrosamine.

Around 75% of cancers of oral cavity and pharynx are caused by the use of tobacco. The association between tobacco and cancer becomes more pronounced with increased amount of tobacco consumption, either in the form of smoking or

chewing, and also duration of consumption. In India, the per capita consumption of cigarettes has seen an increase by 2% over the last decade. Estimates for the annual number of persons developing disease attributable to their tobacco habit in mid-eighties included 1,08,000 annual incident cases of cancer (Brennan and Boffetta 2004). The first report of an association between tobacco smoking and lung cancer risk came in 1950 (Wynder and Graham 1950; Doll and Hill 1950) and over the subsequent years, several studies not only confirmed this finding but also found that tobacco smoking also increases the risk of several other neoplasms. In 2004, International Agency for Research on Cancer (IARC) classified tobacco smoking as carcinogenic to humans and gave evidence for the relationship between cigarette consumption and cancers of several regions of body such as lung, head and neck, stomach, pancreas, liver, kidney, ureter, urinary bladder, cervix, and leukemia. In Indian context, a National survey revealed that smoking tobacco is more prevalent in men while women chewed tobacco more and the regular consumption of tobacco is more prevalent in later stages of life (50 years and more) in both men and women (Neufeld et al. 2005). The recent National Cancer Registry Programme (NCRP) data on Tobacco related cancers (TRC) analyzed in a study conducted by Asthana et al. (2016) revealed that approximately one-fourth of all cancers among men and one-fifth among women were tobacco related. The maximum incidence among men was reported for esophagus, lungs, and hypopharynx cancers from the North-east of India and mouth cancer for Western India. Among women, maximum rates were reported for esophagus and lung cancers in the Northeast region. The overall risk of developing TRC was highest in the Northeast region compared to all other regions. A study which compared the overall incidence of all cancer cases from Northeast region and other regions from India observed a higher cancer incidence in the Northeast. The study also compared Northeast cancer incidence data with global data and found its similarities with the Southeast Asian region. This might be due to the similarities between the genetic pool of people in Northeast region of India and Southeast Asian region (Sharma et al. 2014).

10.2.2 Alcohol and Cancer Risk

Alcohol has been known to be responsible for several health and social issues and is responsible for about three million deaths each year with about 5.1% of the global burden of disease and injury (World Health Organization 2018). Besides being a causal factor for several health issues such as liver cirrhosis, epilepsy, poisoning, etc., alcohol consumption was estimated to have caused around 500,000 cancer deaths worldwide in 2004, 4.4% of cancer deaths in China in 2005 and 3.5% in the USA in 2009. The first study demonstrating the carcinogenic effect of alcohol was published in 1903 (Newsholme 1903) followed by several such studies which paved the way for the International Agency for Research on Cancer (IARC) listing alcohol among the carcinogens for oral cavity and pharynx, esophagus, liver, and larynx. The list was updated to include two more sites colorectum and female breast in 2010 following the studies conducted by Hamajima et al. (2002) and Ferrari et al. (2007).

There are several mechanisms by which alcohol consumption may exert its carcinogenic effect. Acetaldehyde production, polymorphisms in ethanol metabolizing genes, hormonal imbalances leading to breast cancer, dysregulation of immune system, dysregulation of folate metabolism, and damages to the epithelium resulting in increased absorption of carcinogens are some of the mechanisms that promote carcinogenesis (Boffetta and Hashibe 2006; Yu et al. 2010; Singletary and Gapstur 2001; Watson et al. 1994; Hamid et al. 2009; Doll et al. 1999). It has been found that methylation of p15-gene is accelerated by tobacco and alcohol promoting the development of head and neck cancer (Chang et al. 2004) as alcohol and tobacco are likely to be synergistic in causing head and neck cancer (Murata et al. 1996). In studies where alcohol is co-administered with other cancer-causing agents, it has been found to exert a synergistic effect in promoting carcinogenesis (Hsu et al. 1991). This is true for the association between alcohol, smoking and head and neck cancer risk though a study by Schlecht et al. (1999) found the joint effects of alcohol and moderate smoking to be more than multiplicative in pharyngeal cancers. The study pointed out that alcohol has both synergistic and independent effects in promoting carcinogenesis of head and neck region. The synergistic action could be by acting as a solvent for other carcinogens or promoting inflammation and generation of reactive oxygen species. Studies have reported an association between alcohol consumption and pancreatic and prostate cancer. With regard to pancreatic cancer, heavy consumption of alcohol was associated with an increased risk by 19% compared with non-drinkers or occasional drinkers while two studies reported a significant positive association between alcohol consumption and prostate cancer. Alcohol consumption is also associated with colorectal cancer, though gender specific differences do exist as a meta-analysis did not find any significant association between alcohol and colorectal cancer mortality in women (Cai et al. 2014).

The association of alcohol consumption has also been studied with an increased risk of melanoma. The possible mechanisms of alcohol-induced melanoma could be enhanced cellular damage in the presence of UV radiation and subsequently formation of skin cancers (Saladi et al. 2010) and increased immunodeficiency and immunosuppression (Watson et al. 1994), the conditions that facilitate melanoma formation (Mukherji 2013). A meta-analysis conducted by Bagnardi et al. (2015) found that heavy drinkers had a significant 15% increase of lung cancer risk as compared with non-drinkers or occasional drinkers. However, in another meta-analysis published by the same group, alcohol consumption was not associated with lung cancer risk in never smokers. Similarly, an association between alcohol consumption and stomach cancer was also observed. These positive associations must be interpreted with caution as the confounding effect of smoking cannot be ruled out given the fact that alcohol users are most commonly smokers too. Another important consideration is the influence of diet as heavy alcohol consumption leads to compromised nutrition and, thus, confounding by poor diet (Klatsky 2001) could not be ruled out. Inconsistent and inconclusive reports for the association between alcohol consumption and cancers of endometrium, ovary, cervix, thyroid, and brain have been reported though there are also studies which reported no association with

cancer of bladder, adenocarcinoma of the esophagus, and stomach while significant association was observed with cancer of gall bladder (Bagnardi et al. 2015).

As opposed to the studies which show a positive association between alcohol consumption and cancer risk, there are also studies which reported an inverse relationship. Studies on Hodgkin's and non-Hodgkin's lymphomas reported an alcohol-induced decrease in the risk (Tramacere et al. 2012a, b) while protective effect of moderate alcohol consumption on the risk of renal cell cancer has also been reported. However, a European study did not report an inverse relationship between alcohol intake and lymphoid neoplasms (Heinen et al. 2013). The possible reasons for alcohol related decrease in the risk of lymphomas are not very evident though it may be attributed partly to the inappropriate classification of drinkers as non-drinkers as in the initial stages of the lymphomas, the cases either abstain from drinking or reduce the alcohol intake significantly. Similarly, alcohol could protect renal cells from cancer either due to its role in insulin sensitivity or diuresis though no concrete evidence exists between fluid intake and cancer risk (Altieri et al. 2003). Some studies have established an association between alcohol and risk of head and neck cancer. This was particularly evident in populations with a higher alcohol intake as revealed by cohort and case-control studies (Viswanathan and Wilson 2004). All forms of alcohol have been linked to the cancers of oral cavity and pharynx with the association more strong for oral cavity compared to the larynx and pharynx.

Alcohol poses less risk for laryngeal cancer compared to cigarette smoking; however, cancer of the oral cavity has an increased incidence among those who smoke and use alcohol (Choi and Kahyo 1991). The incidence of oral cancer may remain high several years after stopping alcohol use (Franceschi et al. 2000).

10.3 Genetic Variations in Enzymes Involved in Metabolism of Xenobiotics

10.3.1 Genetic Variations of Phase I Xenobiotic Metabolizing Enzymes (XMEs)

The metabolism of Polycyclic Aromatic Hydrocarbons (PAHs), N-nitrosamines, and aromatic amines is by a two-phase process. The first phase called as phase I involves the activation of the carcinogen by enzymes encoded by the CYP gene superfamily. Cytochrome P450s (CYPs) are the most important super family of phase I XMEs which are ubiquitously distributed and found from bacteria to humans (Nelson et al. 1996). The root symbol "CYP" for human ("cyp" for mouse) denotes "cytochrome P450." The root symbol is followed by an Arabic numeral, designating the CYP family (Nebert and Russell 2002). The CYP1, CYP2, and CYP3 enzymes are primarily associated with the metabolism of exogenous compounds, whereas the other CYPs mainly have endogenous roles. It is estimated that CYPs in families 1–3 are responsible for about 75% of all phase I metabolism of clinically used drugs (Evans and Relling 1999). CYPs exhibiting important endogenous functions are well

conserved, while almost all CYPs involved in xenobiotic metabolism are functionally polymorphic (Ingelman-Sundberg 2004). CYP1 family, which consists of CYP1A and CYP1B1, is involved in the metabolic activation of procarcinogens and promutagens to reactive carcinogenic and mutagenic species while CYP2 family which includes CYP2A, 2B, 2C, 2D, and 2E accounts for metabolism of majority of drugs. CYP2E1 is also primarily involved in the metabolism of alcohol and other low-molecular weight compounds and contributes to activation of many procarcinogens and several drugs to highly reactive metabolites (Nakajima and Aoyama 2000). Likewise, CYP2A6 has been specifically demonstrated to activate 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN) tobacco smoke procarcinogens via α-hydroxylation (Patten et al. 1997). CYPs belonging to 2C and 2D family are the major drug metabolizing CYPs and lead to poor or extensive metabolizer phenotype.

CYP1 family members include three genes, CYP1A1, CYP1A2, and CYP1B1. CYP1A1 gene is located on the long arm of chromosome 15 (q22–24) and contains seven exons of which the first is noncoding (Hildebrand et al. 1985). The CYP1A1 m1 polymorphism (CYP1A1*2A) consists of a T-C substitution in the 3′ noncoding region of the gene, m2 polymorphism (CYP1A1*2C) is due to an A to G substitution at nucleotide 4889 in exon 7, m3 polymorphism (T5639C) is found only in African Americans, and m4 polymorphism (CYP1A1*4) leads to a C to A substitution (Cascorbi et al. 1996). While, both CYP1A1*2A and CYP1A1*2C have been reported in Asians, the frequency of CYP1A1*2C is rare in the Caucasians (Hung et al. 2003). CYP1A2 is also located on chromosome 15q22-q24 but shows different substrate specificity from CYP1A1 with a preference for heterocyclic amines, caffeine and a limited number of prescribed drugs including antipsychotics and theophylline (Schmidt 1996). Functionally important polymorphisms in the CYP1A2 are identified in upstream sequence and intron 1 and some of these may affect CYP1A2 protein expression. CYP1A2*1C results from a single nucleotide change from G to A at position −3858 in 5′ flanking region (Nakajima et al. 1999). The frequency of variant allele of CYP1A2*1C is estimated to be around 0.02–0.03 per cent in Caucasians and higher frequency is found in Japanese (0.10) and Chinese population (0.11). The second variant allele is a C to A transversion (CYP1A2*1F) in intron 1 at position 734 downstream of the first CYP1B1, located on chromosome 2, 2p21. Of the most common SNPs of CYP1B1 gene, four have been reported to result in amino acid substitutions including Arg by Gly at codon 48 (CYP1B1*2), Ala by Ser at codon 119 (CYP1B1*2), Leu by Val at codon 432 (CYP1B1*3), and Asn by Ser at codon 453 (CYP1B1*4). A higher catalytic activity for Val432 variants than the Leu432 variants of the enzyme have been reported suggesting that polymorphisms in the human CYP1B1 gene, especially those at codon 432, may contribute to differential susceptibility towards PAH and tobacco-induced cancers (Shimada et al. 1999).

CYP2 is the largest CYP family made up of 13 subfamilies which are involved in drug metabolism in mammals. The genes which code for proteins are CYP2A CYP2B, CYP2C, CYP2D, CYP2E, CYP2F, CYP2J, CYP2R, CYP2S, and CYP2W while CYP2A7 is the pseudogene which produces a hybrid gene with the

CYP2A6 gene (Oscarson 2001). The CYP2A6 gene is 6 kb in size, has 9 exons, and is located within a 350-kilobase pair gene cluster made up of CYP2A7, CYP2A13, two CYP2A7 pseudogenes, and genes in CYP2B and CYP2F subfamilies (Hoffman et al. 1995). CYP2A6 exhibits several genetic polymorphisms such as wild type (CYP2A6*1A), gene conversion with CYP2A7 in the 3′-untranslated region (CYP2A6*1B), gene conversions with CYP2A7 in exons 3, 6, and 8 (CYP2A6*3), L160H (CYP2A6*2), G479V (CYP2A6*5), R128Q (CYP2A6*6), I471T (CYP2A6*7), R485L (CYP2A6*8), and S224P (CYP2A6*11) (Daigo et al. 2002). Other functionally important alleles include CYP2A6*9 which has a point mutation in the TATA box (T-48G), CYP2A6*10 (two simultaneous amino acid substitutions of CYP2A6*7 and CYP2A6*8), CYP2A6*4 (deletion of the whole CYP2A6 gene), and CYP2A6*1X2 (duplication of the CYP2A6 gene). Asian populations (Chinese, Japanese, and Korean) have higher prevalence of CYP2A6 variant alleles resulting in reduced enzyme activity as opposed to the Caucasian and African North American populations (Schoedel et al. 2004). Asian populations have prevalence of whole-gene deletion alleles (CYP2A6*4B and CYP2A6*4C), while European population have higher frequency of CYP2A6*2 and CYP2A6*3.

CYP2E1 metabolizes ethanol, low-molecular weight solvents, and tobacco specific nitrosamines. It is present on the tenth chromosome with 9 exons, 8 introns and has a characteristic TATA box (Guengerich et al. 1991). CYP2E1gene has several polymorphisms majority of which are present in the introns while others are present in 5′-regulatory, intron, and transcribed regions. The polymorphisms studied with restriction enzymes include *PstI* (G to C substitution at -1293 bp in the 5'noncoding region of the CYP2E1gene) and *RsaI* (C to T substitution at −1053 in 5'noncoding region of the CYP2E1) which are in linkage disequilibrium and together called as CYP2E1*5B, with c1 and c2 alleles (Watanabe et al. 1990). c2 allele results in about ten-fold higher gene transcription, protein level, and enzyme activity compared to the c1 allele. Another polymorphism studied using restriction enzyme *XbaI* is CYP2E1*1C having 43–60 bp six repeats and CYP2E1*1D with eight repeats. The intron region of CYP2E1also has several polymorphic sites such as *DraI* which constitutes a T to A substitution at 7668 bp in intron 6 that delete *DraI* restriction enzyme cleavage site and is represented as CYP2E1*6 allele. *DraI* polymorphism has two homozygous genotypes named as CC and DD and one heterozygous genotype, CD (Stephens et al. 1994). Conflicting reports are present regarding the effect of CYP2E1*6 on expression and activity with Persson et al. (1993) observing no effect while Uematsu et al. (1991) reported altered CYP2E1 catalytic activity with CYP2E1*6 allele but no effect on gene transcription. Another polymorphism present in intron 6 region of CYP2E1gene is the *MspI* polymorphism which leads to the A-G replacement at 6827 bp causing deletion of *MspI* restriction enzyme cleavage site. Little information is available about this polymorphism and it does not affect the expression or enzyme activity. There are several polymorphism of *CYP2E1* which are present on coding region and lead to amino acid replacements (Danko and Chaschin 2005). The distribution of CYP2E1 genotypes exhibit ethnic variations with most of the polymorphisms of CYP2E1reported in Caucasians as

Table 10.2 Functionally important polymorphisms in carcinogen metabolizing CYPs

Allele	Protein	Nucleotide change	Effect
CYP1A1			
*CYP1A1*2A*	CYP1A1.1	T3927C	–
*CYP1A1*2C*	CYP1A1.2	A2455G	I462V
*CYP1A1*4*	CYP1A1.4	C2453A	T461N
CYP1B1			
*CYP1B1*2*	CYP1B1.2	C142G; G355T	R48G; A119S
*CYP1B1*3*	CYP1B1.3	C4326G	L432V
*CYP1B1*4*	CYP1B1.4	A4390G	N453S
CYP2E1			
*CYP2E1*5B*	CYP2E1.1	C-1053 T	–
*CYP2E1*6*	CYP2E1.1	T7632A	–
CYP2C19			
*CYP2C19*2*	–	G681A	Splicing defect
*CYP2C19*3*	–	G636A	Premature stop codon
CYP2D6			
*CYP2D6*4*	–	G1846A	Splicing defect
*CYP2D6*10*	CYP2D6.10	C100T; G1661C; G4180C	P34S; S486T
CYP2A6			
*CYP2A6*1B*	CYP2A6.1	A-1013G	Gene conversion in the 3′ flanking region
*CYP2A6*4C*	–	CYP2A6 deleted	CYP2A6 deleted

well as in the Oriental population while only two polymorphisms (*RsaI* and *DraI*) are reported in Indian populations (Soya et al. 2005). Caucasians and Indians have similar prevalence of the minor allele c2 while the Orientals carry a higher frequency. For *DraI* polymorphism (CYP2E1*6), the minor allele frequency is higher in Indian or Oriental (20–30%) populations compared to the Caucasians (7–12%). Table 10.2 lists some of the functionally important polymorphisms in CYPs.

10.3.2 Genetic Variations of Phase II Xenobiotic Metabolizing Enzymes (XMEs)

One of the important classes of phase II xenobiotic metabolizing enzymes is Glutathione S-transferases (GSTs) which play an important role in the detoxification of tobacco carcinogens such as PAH diol epoxides, aromatic amines, hydrazines and products of oxidative stress. Of the five different families of GST, four are cytosolic and one is microsomal. Cytosolic GSTs conjugate glutathione (GSH) with electrophilic substrates increasing their solubility and excretion from the body. In humans, cytosolic GSTs exist in eight classes having one or more of the homodimeric or heterodimeric isoforms (Bolt and Their 2006). GSTs also regulate other physiologically important enzymes and proteins such as that of DNA repair (Huang et al. 2009)

and are therefore important for both detoxification and maintaining cellular genomic integrity.

On the basis of their degree of sequence identity, GSTs have been assigned to eight families designated as Alpha (α), Mu (μ), Pi (π), Sigma (σ), Theta (θ), Zeta (ζ), Omega (ω), and Kappa (κ). The different isoforms such as GSTM1, GSTM3, GSTP1, and GSTT1have been shown to exhibit genetic polymorphisms. GSTM1, GSTM3, and GSTP1are involved in the detoxification of the PAH diol epoxides while GSTT1 detoxifies tobacco smoke components such as monohalomethanes and reactive epoxide metabolites of butadiene. GSTM1 is a member of GSTM family of enzymes and is involved in the detoxification of polycyclic aromatic hydrocarbons, ethylene oxide, epoxides, and styrene (Takahiko et al. 2008). Both the lack of this enzyme and overexpression can have consequences, as deficiency of this enzyme increases the cancer risk due to decreased elimination of carcinogenic compounds (Rebbeck 1997), while overexpression may result in chemotherapeutic resistance. Some factors, such as genetic variations, may alter gene expression or enzyme activity of GSTM1 and have an important bearing on cellular protection from environmental and oxidative stress and response to drugs. GSTM1 gene has 8 exons flanked by two almost identical 4.2-kb regions and GSTM1*0 deletion polymorphism is caused by a homologous recombination process involving both repeats (Xu et al. 1998). Studies focussing on genetic polymorphisms of GSTM1 have compared the homozygous deletion genotype with genotypes having one functional allele. Homozygous gene deletions exhibit variable frequencies in different ethnic groups. GSTM1 has two other alleles designated as GSTM1A and GSTM1B which differ by a C > G substitution at base position 534 resulting in the substitution of Asn > Lys at amino acid 172 (Seidegard et al. 1998) though this results in no functional difference between the two alleles. GSTM1A and GSTM1B are, therefore, categorized together as non-null conjugator phenotypes. Several studies have reported an association between GSTM1*0 (homozygous deletion) and increased risk of cancers and with better treatment outcomes.

The rates of prevalence of null genotypes of GSTM1 are high across several ethnic groups with a meta-analysis of 30 studies (Garte et al. 2001) involving over 10,000 individuals reporting a frequency of 53% for the Caucasians and a similar frequency was seen in Asians but lower in African Americans (16–36%). In the USA, different ethnic groups exhibit differences in frequencies of GSTM1 such as from 23% to 41% for persons of African descent, from 32% to 53% for persons of Asian descent, from 40% to 53% for those of Hispanic descent, and from 35% to 62% for those of European descent. For South American populations, studies have reported frequencies of 21% for Chileans (Quinones et al. 1999), 55% for Caucasian Brazilians, 33% for Black Brazilians, and 20% for Amazonian Brazilians. European populations also exhibit variations in deletion frequency with 46% for French, 53% for Italians, 44% for Hungarians and 50% for the Slovaks. Some groups such as Pacific Islanders and Malaysians have a very high GSTM1 deletion frequency of 62–100%. Similarly, other Asian populations, such as the Japanese and Chinese, have frequencies ranging from 48% to 50% and 35% to 63%, respectively (Rebbeck 1997).

GSTT1 plays a role in detoxification of carcinogens found in tobacco smoke and pesticides, epoxybutanes and ethylene oxide. As opposed to the role of GSTM1, GSTT1 has both detoxification and activation roles as seen in case of activation of dihalomethanes to dichloromethane, which has been shown to cause liver and lung tumors in mice. GSTT1 is different from other members of GST family as it is expressed not only in the adult liver but also in human erythrocytes and thus has a wider role in detoxification of carcinogens in the body (Landi 2000). The GSTT subfamily is made up of two genes, GSTT1 and GSTT2.These are located at 22q11.2 and separated by about 50 kb having five exons each with identical intron/exon boundaries. However, these share only 55% amino acid identity. There is no role of GSTT2 in deletion of GSTT1 (Coggan et al. 1998). GSTT1 has two flanking 18 kb regions named as HA3 and HA5 with >90% homology and having identical 403-bp repeats, which function as deletion/junction regions of the GSTT1 null allele (Sprenger et al. 2000). GSTT1 deletion results from a homologous recombination event involving the left and right repeats causing a 54-kb deletion containing the entire GSTT1 gene. Ethnic variations have been reported regarding the distribution of null allele of GSTT1. Studies of GSTT1 null genotype demonstrate that 20% of Caucasians, 47–64% of Asians, 15–31% of Europeans and 22–29% of African Americans have deletion genotype of GSTT1. Asian populations have greater prevalence of GSTT1 compared to the Europeans as shown by studies which report 21% of Italians and 28% of Slovakians have deletion while 58% of Chinese, 38% of Malaysians (Lee et al. 1995), 42% and 46% Koreans have GSTT1 deletion genotype.

GSTP1 is involved in the metabolism and detoxification of many carcinogenic xenobiotics such as diol epoxides of polycyclic aromatic hydrocarbons (PAHs). GSTP1 has been the focus of several studies because of its over-expression in pre-neoplastic and tumor tissues which makes it useful as an early tumor marker, and is also responsible for drug resistance of many cancers. GSTP1 gene is located at 11q13 and is 2.8 kb long with seven exons (Morrow et al. 1989). The open reading frame starts at the 3′ end of the first exon and is 630 bp long, encoding a protein of 209 amino acids. GSTP1 has two common non-synonymous SNPs that result in Ile105Val and Ala114Val alterations in encoded amino acid sequence and are associated with variations in cancer risk and treatment response (McIlwain et al. 2006). The effect of Val105 substitution is due to steric restriction of the H-site due to shifts in the side chains of several amino acids which leads to less accommodation of less bulky substrates than the Ile105 allozyme (Johansson et al. 1998). Moreover, codon 105 variant allozyme has different thermal stability compared to the wild type (Johansson et al. 1998). These are the probable reasons for the use of GSTP1 as an early tumor marker and in pharmacogenetics. As seen with GSTM1 and GSTT1, ethnic variations have also been reported in the distribution of variant genotypes of GSTP1. In Africa, the frequency of GSTP1 Val105 variant has been reported to be 14% among South Africa, 16%, 12%, and 21% among Tanzanians, South African Venda, and Zimbabweans, respectively (Dandara et al. 2002) and 53% (The Gambia) (Wild et al. 2000). Caucasians have an allele frequency of 28–38%, while Asians have 16–26%. Another polymorphism of GSTP1, A114V, is less

Table 10.3 Functionally important polymorphisms in GSTs

GST class	Allele	Nucleotide change(s)	Protein change(s)
Mu (μ)	*GSTM1*A*	534G	Lys172
	*GSTM1*B*	534C	Asn172
	*GSTM1*0*	Gene deletion	No protein
	*GSTM1*1x2*	Gene duplication	Protein over-expression
	*GSTM3*A*	Wild type	–
	*GSTM3*B*	3 bp deletion in intron 6	None
	*GSTM4*A*	Wild type	–
	*GSTM4*B*	T2517C	None
Theta (θ)	*GSTT1*A*	Wild type	–
	*GSTT1*0*	Gene deletion	No protein
	*GSTT2*A*	415A	Met139
	*GSTT2*B*	415G	Ile139
Pi (π)	*GSTP1*A*	Wild type	–
	*GSTP1*B*	313G	Val105
	*GSTP1*C*	313G, 341 T	Val105, Val114

well studied and the variant allele is usually found in linkage disequilibrium with GSTP1 I105V. This polymorphism has not emerged as a prominent genetic variation of GSTP1 owing to its lower frequency and no effect on enzyme activity. It also exhibits a lower frequency of 5% in African Americans and 9% in European Americans (Watson et al. 1998). Table 10.3 lists some of the functionally important polymorphisms in GSTs.

10.4 Gene–Environment Interactions and Cancer Risk

10.4.1 Gene–Environment Interaction: Model and Issues

With rapid developments in the field of molecular biology, we now have a better understanding of the effect of genetic factors on human diseases though the genetic influences are exceedingly complex. Both genetic and environmental factors have role in determining the susceptibility to diseases and the study of gene–environment interaction is the key in genetic epidemiology. As for the interaction between any two risk factors for a disease, gene–environment interaction also faces the same complications such as the source of the interaction and its detection. Ottman (1996) provided possible models of relations between a genotype and an environmental exposure in terms of their effects on disease risk. In Model A, the effect of the genotype is to enhance the effect of a "risk factor" that can also be produced nongenetically, in Model B, the genotype worsens the effect of the risk factor, but there is no effect of the genotype in unexposed persons, in Model C, the exposure worsens the effect of the genotype, but there is no effect of the exposure in persons with the low-risk genotype, in Model D, both the exposure and the genotype are

required to increase risk, and in Model E, the exposure and the genotype each have some effect on disease risk, and when they occur together risk is higher or lower than when they occur alone. For testing these models, individuals must be categorized based on the presence or absence of both the exposure and the high-risk genotype. Several strategies can be employed for testing these models such as testing for an identified susceptibility gene, measurement of candidate genes or ecogenetic markers (Perera and Whyatt 1994), using a genetic marker that is associated with the disease as a surrogate for the high-risk genotype (Saunders et al. 1993), linking of agenetic marker to a disease susceptibility gene, though the actual disease-causing mutation has not been identified and use of family history data as a surrogate for the genotype.

In the case of cancers, the model assumes that there is an association between cancer and the environmental factors such as tobacco smoking, and without the exposure, the genetic risk factors do not have a role in disease development. The genetic factors can modulate the association between exposure and cancer and in case of tobacco smoke, the relationship becomes more complex as the interaction is not linear but has a dose response (Taioli et al. 1998). There are also other important factors which need to be considered in taking smoking as an exposure. Some important factors are age when smoking started, smoking amount, type, duration, and number of quitting attempts (Vineis 2007). In case of breast cancer, age of starting smoking is crucial as young breast tissue is more prone to carcinogenesis compared to the more matured tissue. Moreover, age also plays a role in females as the sex hormone levels vary according to the age due to which the interaction between smoking and estrogen levels may play a significant role. The other important factor to consider is the interplay between causative factors of different cancer types and their interaction. For example, there is an effect of smoking on cancers which are caused by changes in hormone levels as tobacco smoking inhibits the aromatization of androgens into estrogens. Another area which also needs careful attention is epigenetic modifications. Environmental factors may trigger epigenetic changes which are independent of genetic variations as seen in case of hypermethylation of metabolic and DNA repair genes in several cancers (Russo et al. 2005). Studies have reported an association between methylation of genes and smoking, and, interestingly in one case, methylation of the p16 gene promoter was associated with early onset of smoking (Jarmalaite et al. 2003; Chang et al. 2004).

10.4.2 Interaction of Genetic Variations with Tobacco

Several studies have been conducted to decipher the association between genetic variations and cancer in relation to tobacco consumption. A number of meta- and pooled analysis have been done to study the association between several Phase I and Phase II metabolic gene polymorphisms and lung cancer (Schwartz et al. 2007). A study conducted by Singh et al. (2010) reported an increased risk of lung cancer in smokers who carried variant genotypes of CYP1A2. Similarly, an increased risk to lung cancer in cases who were regular tobacco chewers and carried variant

genotypes of CYP1A2*1D or CYP1A2*1F was also observed in the study. Since CYP1A2 is induced by smoking and is involved in the metabolic activation of heterocyclic amines and procarcinogens (Landi et al. 1999; Bofetta et al. 2008), there is a strong interaction of smoking with polymorphic CYP1A2 genotypes in enhancing the risk to lung cancer. Other studies have also provided evidence for interaction of CYP1A1 and 1B1 genotypes with tobacco chewing in enhancing the susceptibility to lung cancer (Shah et al. 2008a, b). Apart from lung cancer, an elevated risk was also observed in smokers who carried variant alleles of CYP1A2*1D and CYP1A2*1F in pancreatic cancer (Li et al. 2006).

The Gallbladder cancer is a deadly malignancy which exhibits considerable differences in prevalence among certain ethnicities and geographic regions. The areas which are the most affected regions are the Indo-Gangetic plains of India, Mapuche Indians in Chile and South America (Sharma et al. 2017). A meta-analysis showed that GSTM1 deletion and NAT2 slow acetylator status were significantly associated with bladder cancer and an interaction with smoking was only observed for the NAT2 polymorphisms (García-Closas et al. 2005). Studies have also been carried out in the Indian population to study the role of genetic variations in the development of gall bladder cancer. A case-control genome-wide association study of gallbladder cancer cases and controls of Indian descent observed genome-wide significant associations for ABCB1 and ABCB4 genes which suggests the importance of hepatobiliary phospholipid transport in the pathogenesis of gallbladder cancer in Indian populations (Mhatre et al. 2017). Another study performed in a north Indian population showed significant associations of the SNPs in TERT (rs2736100C > A, CLPTM1L rs401681C > T and CASC8 rs6983267G > T) with gallbladder cancer risk (Yadav et al. 2018). Several other studies by Mittal and co-workers have also reported the association of polymorphisms in several genes with risk of gallbladder cancer in north Indian population. Genetic polymorphisms of IL-1 (Vishnoi et al. 2008), Complement receptor 1 (Srivastava and Mittal 2009), OGG1 and XRCC1 (Srivastava et al. 2009), ERCC2, MSH2, and OGG1 (Srivastava et al. 2010a), Caspase-8 (Srivastava et al. 2010b), CYP7A1 (Srivastava et al. 2010c), ADRβ3 T190C (Rai et al. 2014), and TERT-CLPTM1L and 8q24 (Yadav et al. 2018) were reported to be associated with gallbladder cancer risk. In an another gene–environment study in north Indian population, usage of tobacco (smoking or nonsmoking) by gallbladder cancer patients showed a significant increase in cancer risk with CYP1A1 Msp1 polymorphism (Pandey et al. 2008).

Studies conducted on HNSCC reported an association between tobacco use and genetic variations of CYP1A1 (Singh et al. 2009). Amount of tobacco use also influences the outcome of such interaction as a study in a Japanese population reported that the risk associated with CYP1A1*2A genotype is inversely proportional to the amount of tobacco use (Sato et al. 1999; Tanimoto et al. 1999) and Sato et al. (2000) observed that the risk was highest in the group with the lowest cigarette dose level. Variant genotypes of CYP1B1*2 were found to interact with cigarette smoking while no such significant interaction was reported with CYP1B1*3 or CYP1B1*4 (Li et al. 2005; Singh et al. 2008b). However, contrasting reports are also available with Ko et al. (2001) reporting that variant genotypes of CYP1B1*3

significantly interact with smoking and smokers with the variant genotypes were 20 times more likely to show evidence of p53 mutations than those with a CYP1B1 wild type genotype. As observed with tobacco smoking, significant gene–environment interaction was observed with variant genotypes of CYP1B1*2 and CYP1B1*3 and tobacco chewing (Singh et al. 2008b). Studies also show a positive correlation of variant genotypes of CYP2C19 with tobacco use which could be possibly attributed to the lower ability of cases with genetic variations for detoxifying the carcinogens generated by tobacco chewing or smoking (Sugimoto et al. 2005). In contrast, CYP2D6 genotypes did not appear to interact significantly with tobacco (Caporaso et al. 2001).

A study by Ruwali et al. (2009a) suggested that polymorphic CYP2E1 genotypes interact with tobacco and increase the HNSCC risk with a greater increase in risk observed in tobacco chewers which may be due to enhanced formation of nitrosamines in tobacco chewing (Hecht and Hoffmann 1988). RsaI polymorphism leads to a greater increase in transcription or enzyme activity than the DraI genotypes due to which stronger association was observed with RsaI (Uematsu et al. 1994). Soya et al. (2008) also reported an interaction between CYP2E1*6 (DraI) genotypes and tobacco among South Indian tobacco users for upper aerodigestive tract cancers. In addition, Harth et al. (2008) found an interaction of CYP1B1 (Leu432Val) and CYP2E1 (−70G > T) genotypes among smokers indicating the relevance of combined genotypes with exposure to tobacco smoke in significantly enhancing the HNSCC risk. In contrast, another study by Ruwali et al. (2009b) reported a reduction for HNSCC risk in individuals who are exposed to risk factors such as tobacco consumption (in the form of smoking and chewing) when compared to individuals who are not exposed to such risk factors and carrying the variant genotypes of CYP2A6. This reduction in HNSCC risk may possibly be attributed to a higher number of cases with variant genotypes of CYP2A6 to be non-tobacco users than tobacco users as variant alleles of CYP2A6 have been reported to reduce the risk of tobacco consumption in the form of smoking and thus it is smoking that results in a decreased HNSCC risk.

Among the phase II xenobiotic metabolizing enzymes, several studies have been carried out to investigate the association between genetic variations in GSTs and tobacco use. Studies have reported an association between null genotypes of GSTM1 or GSTT1 and tobacco use in head and neck cancer patients (Sabitha et al. 2008; Singh et al. 2008a; Ruwali et al. 2011). Smoking intensity also plays an important role in deciding the outcome of the interaction. A significant association of GSTM1 null genotype with laryngeal cancer risk in light smokers was observed (Jourenkova et al. 1998), while in some studies GSTM1 or GSTT1 null genotype was found to be linked to upper aerodigestive tract (UADT), non-laryngeal UADT or oral cancer risk in heavy smokers. Among the reasons for such an interaction could be the higher sister chromatid exchange (SCE) and chromosomal aberration levels in smokers with GSTM1 null genotype than GSTM1 positive smokers (Nora 2004). In contrast to these studies, some of the earlier studies failed to find any significant interaction of GST genotypes with tobacco-induced oral and pharyngeal cancer (Jourenkova-Mironova et al. 1999). For GSTP1, inconsistent

reports are available regarding the relationship between the GSTP1 polymorphisms and smoking status in HNSCC though the variant forms of GSTP1 are more efficient in detoxifying the carcinogenic epoxide of benzo(a)pyrene with high and selective activity (Saarikoski et al. 1998).

10.4.3 Interaction of Genetic Variations with Alcohol

Similar to tobacco use, significant interaction has been reported between alcohol and genetic variations in phase I and phase II xenobiotic metabolizing enzymes. Heterozygous or homozygous genotype of CYP1B1*2 either alone or in combination resulted in high (five to sixfold) or very high (about 20 fold) increase in the risk to HNSCC (Singh et al. 2008b) though alcohol was not found to interact with CYP1A1 genotypes (Singh et al. 2009). Studies have further shown that individuals with variant genotypes of CYP2C19*2, CYP2C19*3, CYP2D6*4 and who were regular alcohol users were at several fold increase in the risk to HNSCC when compared to the controls, who were regular alcohol drinkers (Yadav et al. 2008, 2010). Similarly, alcohol consumption was also found to interact with variant CYP2E1 genotypes as the risk associated with RsaI variant genotypes and DraI variant genotypes was significantly higher in the HNSCC cases who were regular alcohol users when compared with the controls (Ruwali et al. 2009a). However, a study by Singh et al. (2010) found no significant increase in risk to lung cancer in alcohol users among cases with variant genotypes of CYP1A2 when compared to non-alcohol users. A study by Shah et al. (2008a) reported about 5-6 fold increase in the risk for lung cancer in the alcohol users with the variant genotypes of CYP1A1*2A and CYP1A1*2C. In a pooled analysis of cohort studies, it was found that alcohol consumption was associated with increased risk to lung cancer in male never smokers (Freudenheim et al. 2005). Since people who smoke are also alcohol users, the major concern in the examination of an association between alcohol consumption and lung cancer has been the failure to control for confounding by smoking.

Among the phase II xenobiotic metabolizing enzymes, several studies have been carried out to investigate the association between genetic variations in GSTs and alcohol use. A higher increase in HNSCC risk was observed in cases who were regular alcohol users and carried null genotypes of GSTM1 or GSTT1 compared to non-alcohol users (Singh et al. 2008a). Similarly, strong association in regular alcohol users carrying GSTT1 null genotype and a higher risk for multiple primary neoplasmas in UADT cancers was also reported (Soya et al. 2007). The role of alcohol–tobacco interaction in HNSCC risk was investigated by Peters et al. (2006) in a study which showed that deletion of GSTM1 markedly increased the alcohol–tobacco interaction. Homozygous deletion of GSTM1, tobacco, and alcohol exhibit a tri-modal interaction with the risk being higher among those who were both heavy smokers and low alcohol consumers with the possibility that alcohol may be facilitating the entry of tobacco carcinogens into oral tissues (Howie et al. 2001). Interestingly, there is also report that variants in certain genes lower the risk of

various types of cancers in individuals who consume alcohol. One such gene is alcohol dehydrogenase (ADH) which is involved in the metabolism of alcohol. A study by Hashibe et al. (2008) analyzed six ADH variants in more than 3800 individuals with aerodigestive cancer and over 5200 controls. One variant each in ADH1B and ADH7 was significantly protective against aerodigestive cancer specifically in individuals who were alcohol drinkers, and most strongly in those who had higher alcohol intake. Individuals with the protective variant in ADH1B are known to metabolize alcohol up to 100 times faster than those without it, suggesting that lower exposure to alcohol is protective against the disease.

10.5 Conclusions

Environmental risk factors such as tobacco (in the form of smoking or chewing) or alcohol use increase the cancer risk in cases carrying the variant genotypes of phase I and phase II XMEs compared to those not exposed to these risk factors. Cancer is caused by certain changes in genes that control the functions of the cell related to growth and division. Cancer-causing gene mutations either occur over the course of a lifetime or are inherited from parents. Studies suggest that genetic variations in genes metabolizing tobacco and alcohol interact with these environmental risk factors and increase the cancer risk clearly stating the significance of gene–environment interactions. The studies indicate that gene–environment studies have a higher power than only environmental studies. The applications of gene–environment studies range from searching for new causes of disease (when the effects of the tested genes, environmental exposures or both are unknown), or exploring the mechanisms of cellular action of established environmental factors, such as smoking. Such studies need to assemble large data sets and large well-characterized populations in order to test complex gene–environment interaction pathways. This will help in improving accuracy and precision in the assessment of both genetic and environmental influences leading to well-informed recommendations for cancer prevention.

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Sequestration of Carbon Dioxide by Microorganism and Production of Value Added Product

11

Randhir K. Bharti, Shaili Srivastava, and I. S. Thakur

Abstract

The rising carbon dioxide (CO_2) emission leading to global climate change is one of the greatest environmental challenges that the world faces today. The link between the anthropogenic CO_2 emissions and its increased atmospheric concentration resulting in global average temperature rise and consequent sea level rise is well established. The CO_2 mitigation can be achieved by three means: first by improving energy efficiency, second by CO_2 capture and sequestration, and the third option is use of alternative clean fuels (biohydrocarbon, biodiesel, etc.). The most important global carbon sinks are green plants, algae, and some photosynthetic and chemolithotrophic bacteria. Some microbes fix CO_2 with the help of special enzymes such as carbonic anhydrase, Rubisco, and other carboxylases. These include the Calvin cycle, reductive tricarboxylic acid cycle, Hydroxypropionate–hydroxybutyrate cycle, Dicarboxylate–hydroxybutyrate cycle, and 3-hydroxypropionate pathway. Calvin cycle is the most prominent cycle found in the autotrophic organism and Rubisco is the key enzyme for CO_2 fixation. In this scheme, the sugar bisphosphate ribulose-1, 5-bisphosphate (RuBP) serves as the acceptor molecule for CO_2, with the enzyme Rubisco catalyzing the actual primary CO_2 fixation reaction. Carbonic anhydrase (CA) is a zinc-containing enzyme that catalyzes the reversible dehydration of HCO_3^- to CO_2. Here the CA functions to convert an accumulated cytosolic pool of HCO_3^- into CO_2 within the carboxysome. It can assist in elevating CO_2 concentrations around Rubisco. Some microbes synthesize valuable products such as different types of alkanes/alkenes/Lipids/TAG which can be utilized for

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biofuel production after sequestration of CO_2. Biodiesel is produced from TAG by transesterification reaction in the presence of methanol and catalyst.

11.1 Introduction

Carbon is found everywhere on Earth. Some carbon from the Earth is deposited inertly within the lithosphere of the Earth. The rest of the carbon is contained within the sort of carbon dioxide within the atmosphere (2%), biomass in plants and soils (5%), fossil fuels during a number of geological reservoirs (8%), and as ion collection within the ocean (85%) (Schlesinger 1999).

Earth's temperature has risen by 0.8 °C during the nineteenth century (IPCC 2007) and is anticipated to grow by 1.5–5.8 °C during the twenty-first century (IPCC 2001). The speed of increase in global temperature has risen to 0.15 °C per decade since 1975. Additionally to the 15–23 cm increase in sea level during the twentieth century (IPCC 2007) there was a dramatic change within the environment (Greene and Pershing 2007). Climate change occurs due to emission of greenhouse gas pollution (GHGs) by man-made activities like the change of land-use, burning of biomass, draining of wetland, combustion of fossil fuels and deforestation. Draining of wetlands, soil cultivation deforestation, and fossil fuel combustion. The carbon dioxide concentration increases from 280 ppmv in 1850 to 380 ppmv in 2005 and currently rising of 1.7 ppmv per year (0.46 per cent per year) (IPCC 2007). Methane (CH_4) and nitrous oxide (N_2O) concentrations also grew steadily over the same time (IPCC 2001, 2007). There are three strategies for the lowering of CO_2 discharges to improve climate change: (1) decreasing the global energy use, (2) developing low or no-carbon fuel, and (3) sequestration of CO_2 from point sources or atmosphere through natural and engineering techniques. Manmade emission of CO_2 are estimated at 270 ± 30 Pg by combustion of fossil fuel and 136 ± 30 Pg by land-use change, deforestation, and soil cultivation between 1850 and 1998 (IPCC 2001). In this time, 7 Pg C $year^{-1}$ is discharged by fossil fuel combustion and 1.6 Pg C $year^{-1}$ by land-use change, deforestation, and soil cultivation. Of the total manmade emissions 8.6 Pg C $year^{-1}$, 3.5 Pg C $year^{-1}$ were used up by the atmosphere, 2.3 Pg C $year^{-1}$ by the ocean and the remainder by an unidentified terrestrial sink possibly in the Northern Hemisphere (Tans et al. 1990).

11.2 Carbon Sequestration

Carbon sequestration is the process where inorganic carbon (carbon dioxide) is reduced to organic compounds by living organisms. The most common example is photosynthesis. Autotrophs are those organisms which grow by the fixation CO_2 like plants. Heterotrophs are those organisms which grow on autotrophs.

Sunlight is essential for existence of life on earth. However, complex communities can be generated by microbial chemosynthesis. Chemolithoautotrophic

bacteria are those microbes which consume chemicals from the rock as an energy source and create their own food (Atomi 2002). Chemolithotrophic microorganism occurs in dark habitats like hydrothermal vents, areas of the deep terrestrial subsurface, like caves and area where low organic carbon is present and no competition with photosynthetic organisms occurs. In nutrient deficient habitats, the bacteria fix CO_2 into organic compound through the oxidation of inorganic compounds such as iron, sulfur, and manganese, and supply the energy to higher trophic levels (Madigan et al. 2003).

11.3 Microbial Precipitation of Calcium Carbonate

Most of the bacterial species are capable to precipitate carbonates in an alkaline environment rich in Ca^{2+} ions. Carbonate precipitation by bacteria is a complex process which was described by (Castanier et al. 2000). Carbonate precipitation is associated with metabolic pathways like photosynthesis, nitrogen, and sulfur cycles, and ion exchange (Ca^{2+}/H^{+}). Some researcher found that carbonate metabolism also identified six genes which involved in crystal formation in *Bacillus subtilis* PB19 (Barabesi et al. 2007). The calcium carbonate precipitation by bacteria has been described for involvement in positively charged Ca2+ ions and negatively charged bacteria Cell walls (Hammes and Verstraete 2002). These Ca^{2+} ion-cell wall interactions produce changes in the overall charge of the cell wall. This process permitting interaction between differently charged bacteria. Due to changes in the overall ionic charge, bacteria aggregate to increase the size of the biomineral, and in turn, bacteria become the nucleus of the biomineral (Ferrer et al. 1988; Rivadeneyra et al. 1996, 1998). Figure 11.1 described the typical environment of calcium carbonate precipitation which contain high extracellular calcium concentrations (compared to intracellular) and low extracellular related to intracellular proton concentrations (as a result of alkaline condition). The combination of an extracellular alkaline pH and calcium ions poses an expectedtense environment for bacteria: passive calcium influx as a result of the complementary $Ca^{2+}/2H^{+}$ electrochemical gradients will lead to intracellular calcium intake and excessive proton expulsion (Dania et al. 2009) (Fig. 11.1a).

Survival under such conditions requires active export of intracellular calcium. The ATP dependent calcium pumps, which would decrease intracellular calcium ions and at the same time compensate the proton loss (Fig. 11.1, second b). The latter event could result in a localized increase in pH, due to proton uptake, in the same region as the calcium ion increase, which would procedure an ideal localized precipitation micro environment (Fig. 11.1, section c). The formation of localized micro-environments, survival of the organism is dependent on active calcium metabolism. The energy (ATP) is required for the metabolism of organic substance and produce CO_2 as a by-product which is used as a carbonate. The bacteria will lead to increase in the extracellular dissolve inorganic carbon for the survival and proliferation which would affect the solubility of $CaCO_3$, which also service the precipitation on calcium carbonate (Hammes and Verstraete 2002; Dania et al. 2009).

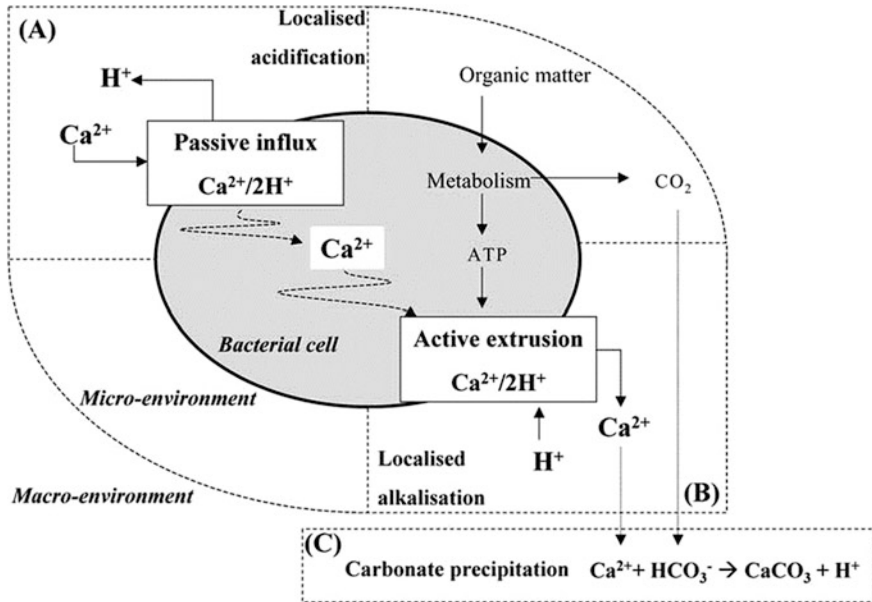


Fig. 11.1 Schematic representation of suggested bacterial calcium metabolism and subsequent $CaCO_3$ precipitation under high-pH and high-Ca^{2+} extracellular conditions (Hammes and Verstraete 2002)

Bacteria produces extracellular polymeric substances (EPS) which have also been exposed to be involved in the method of biocalcification by capturing and serving as a nucleation site. Calcium carbonate formation was occur through the activity of a peptide matrix in Pseudomonas fluorescens, and the major contributing amino acids like glutamic acid, alanine, aspartic acid, and glycine. The change composition and concentration of EPS have an impact on both the morphology and the mineralogy of the carbonate crystals (Braissant et al. 2003). EPS also seems to be involved in increasing the size of the crystals by causing the aggregation of smaller crystals (Dania et al. 2009).

11.4 Metabolic Pathways of Chemolithotrophic Bacteria/ Autotrophic Bacteria

Some microorganisms play an essential role in ecosystems by providing a continuous supply of organic carbon for heterotrophs. The Calvin–Benson–Bassham cycle (Calvin cycle) represents the fundamental autotrophic (chemotrophic or phototrophic) carbon fixation pathway. There are several CO_2 fixation pathways found in microorganism which is present in extreme environments such as high temperature or anaerobic or acidic conditions. (1) Reductive cabroxylic acid (TAC) cycle, (2) the reductive acetyl coenzyme A pathway, (3) the 3-hydroxypropionate

cycle, (4) 3-Hydroxypropionate cycle, (5) Hydroxypropionate–hydroxybutyrate cycle, (6) Dicarboxylate–hydroxybutyrate cycle (Atomi 2002; Madigan et al. 2003). These are the pathways which sequester carbon dioxide.

11.4.1 Calvin Cycle

It is the major and most abundant pathway for CO_2 fixation. This cycle works in some aerobic or facultative anaerobic Proteobacteria, green sulfur bacteria, genera Sulfobacillus, algae, cyanobacteria, and plants. In this cycle, carbon dioxide reacts with the ribulose 1,5-bisphosphate in the presence of Rubisco enzyme to yield two molecule of 3-phosphoglycerate, from which the sugar is regenerated (Calvin and Massini 1952). In this pathway, several enzymes are involved but the key enzymes and CO_2 fixing enzyme is Rubisco.

11.4.2 Reverse Krebs Cycle

This cycle occurs in green sulfur bacterium *Chlorobium limicola*. The complex carbon molecules of Krebs cycle is acetyl CoA which oxidized to form CO_2 and water. In the reverse Krebs cycle takes CO_2 and water to make complex organic compounds acetyl CoA (Evans et al. 1966). Although several enzymes are involved in this pathways but 2-Oxoglutarate synthase, 2-Oxoglutarate synthase and ATP-citrate lyaseare are some key enzymes. The CO_2 fixing enzymes are isocitrate dehydrogenase, pyruvate synthase and carboxylazing PEP (Fig. 11.2) (Buchanan and Arnon 1990; Berg et al. 2010).

11.4.3 Reductive Acetyl Coenzyme A Pathway

In this pathway one molecule of CO_2 is reduced to CO and another one is reduced to a methyl group (bound to carrier); consequently. Acetyl-CoA is synthesized from CO and the methyl group. This is the most energetically autotrophic carbon fixation pathway (Ragsdale 2008). This cycle occurs strictly in anaerobic species which includes proteobacteria, planktomycetes, spirochaetes and euryarchaeota. In this pathway the key enzymes and CO_2 fixing enzymes are Acetyl-CoA synthase and CO dehydrogenase (Berg et al. 2010).

11.4.4 Hydroxypropionate Cycle

This cycle is found in only some green non-sulfur bacteria *Chloroflexus aurantiacus* (family Chloroflexaceae) (Fig. 11.3). The pathway has not been found elsewhere. In this cycle Acetyl-CoA reacts with HCO_3^- in the presence of ATP to form malonyl-CoA which is reduced to 3-Hydroxypropionate. Then converted to four carbon compound succinyl-CoA, which is oxidized to malyl-CoA. Finally, the cleavage

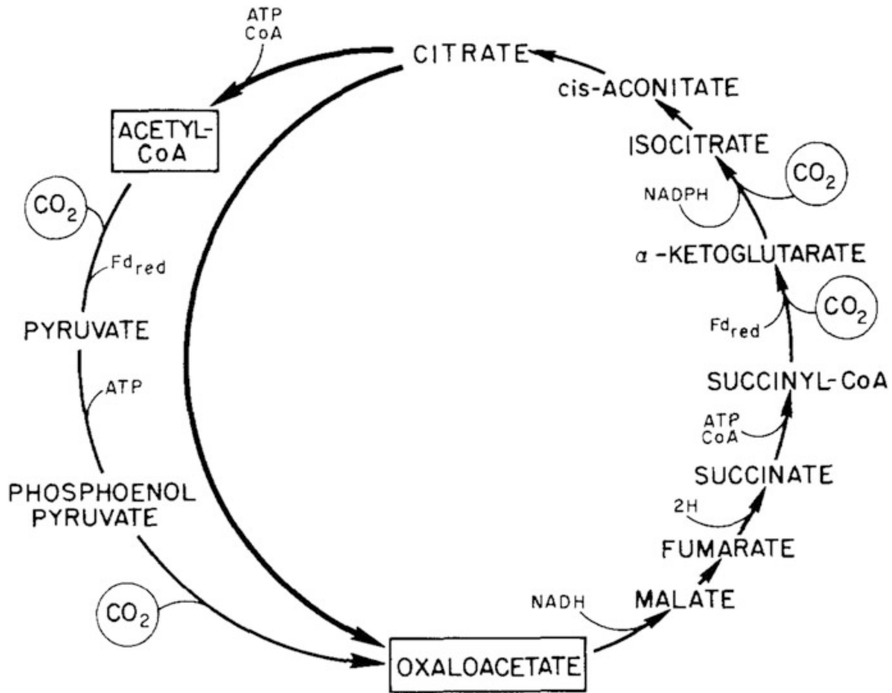


Fig. 11.2 Framework of the Reverse Krebs cycle for autotrophic CO_2 fixation

of malyl-CoA to regenerate acetyl-CoA helps in formation of glyoxylate. The assimilation of glyoxylate requires a second cycle (Berg et al. 2010; Herter et al. 2002).

11.4.5 Hydroxypropionate–Hydroxybutyrate Cycle

This cycle is found in aerobic crenarchaeota and sulfolobales (Strauss and Fuchs 1993). In this pathway, various intermediates and the carboxylation reactions are similar to the 3-hydroxypropionate cycle (Berg et al. 2007). In this pathway, several enzymes are involved but the key enzymes are acetyl-CoA carboxylase, propionyl-CoA carboxylase, methylmalonyl-CoA mutase and 4-hydroxybutyryl-CoA dehydratase and CO_2 fixing enzymes acety-CoA and propionyl-CoA carboxylase (Fig. 11.4).

This pathway is found in anaerobic bacteria like crenarchaeal orders thermoproteales and desulfurococcales (Berg et al. 2010). In these pathways, several enzymes are involved but the enzymes are 4-hydroxybutyryl CoA dehydratase and CO_2 fixing enzymes are pyruvate synthase and PEP carboxylase.

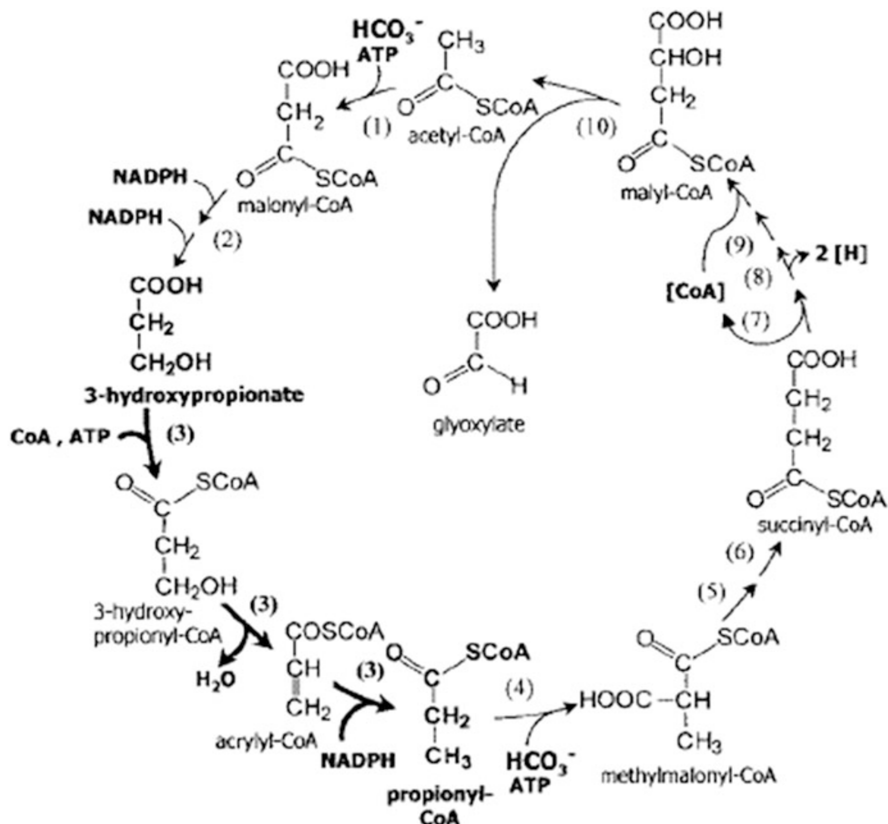


Fig. 11.3 3-Hydroxypropionate cycle of green non-sulfur bacterium *C. aurantiacus.* Number indicates the name of the enzyme. 1, acetyl-CoA carboxylase; 2, malonyl-CoA reductase, a bifunctional enzyme described elsewhere (15); 3, propionyl-CoA synthase, the trifunctional enzyme described in this work; 4, propionyl-CoA carboxylase; 5, methylmalonyl-CoA epimerase; 6, methylmalonyl-CoA mutase; 7, succinyl-CoA:L-malate CoA transferase; 8, succinate dehydrogenase, electron acceptor unknown; 9, fumarase; 10, L-malyl-CoA lyase

11.4.6 Carbon Concentration Mechanism and the Carboxysome

Carboxysomes are proteinceous microcompartments looks like icosahedral and its diameter is 800–1500 A (Yeates et al. 2008). The carboxysomes shell is made up of hexameric protein with 20A thickness and diameter of pore is 4–7 A (Yeates et al. 2008). Rubisco and carbonic anhydrase are tightly packed inside carboxysomes. (Badger and Bek 2008). In this time, there are two known types of carboxysomes. α-carboxysomes and β-carboxysomes. α-carboxysomes are present in oceanic cyanobacteria and in proteobacteria. They include form IA Rubisco and β-carbonic anhydrase (Peña et al. 2010; Yeates et al. 2008). β-carboxysomes found in freshwater cyanobacteria and it have form IB Rubisco, β-carbonic anhydrase, γ-carbonic anhydrase, or a combination of both (Peña et al. 2010).

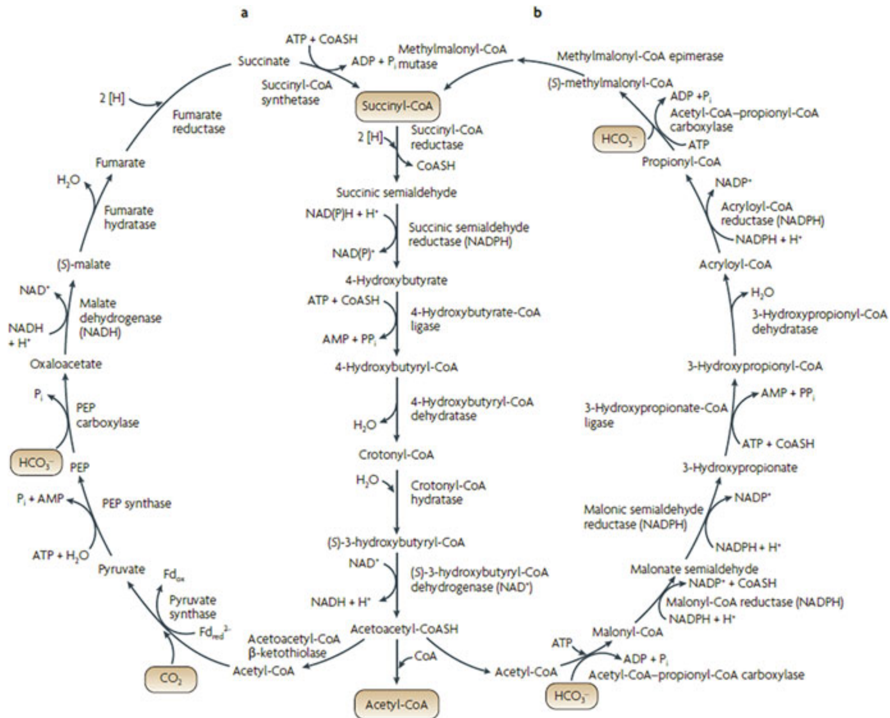


Fig. 11.4 Hydroxypropionate–hydroxybutyrate cycle and Dicarboxylate–hydroxybutyrate cycle (Berg et al. 2010) 2.3.6 Dicarboxylate–hydroxybutyrate cycle

In the ecosystems some bacteria like obligate autotrophs, facultative autotrophs, chemoautotrophs and chemolithoautotrophs which can fix atmospheric CO_2 by the help of two enzyme Rubisco and Carbonic anhydrase which is present in the carboxysomes (a polyhedral organelle) in bacterium. Within the carboxysome, the carbonic anhydrase converts HCO_3^- into CO_2 and the Rubisco fix the captured CO_2 inside the organelle (Yeates et al. 2008, Fig. 11.5). There are several groups of carbonic anhydrases (e.g. α, β, and γ) and several types of Rubisco found in prokaryotes (Peña et al. 2010). The affinity of Rubisco with CO_2 is low, but carbonic anhydrase facilitate CO_2 environment around the Rubisco inside carboxysome. Therefore rate of CO_2 fixation inside the carboxysomes increases.

11.4.7 Carbonic Anhydrase

Carbonic anhydrase is a zinc-containing enzyme that catalyzes the reversible reaction of HCO_3^- to CO_2. The reaction is considered to play necessary roles in numerous biological processes such as respiration, pH homeostasis, ion exchange, CO_2 acquisition, and photosynthesis (Badger and Price 2003). $HCO_3^-$is the

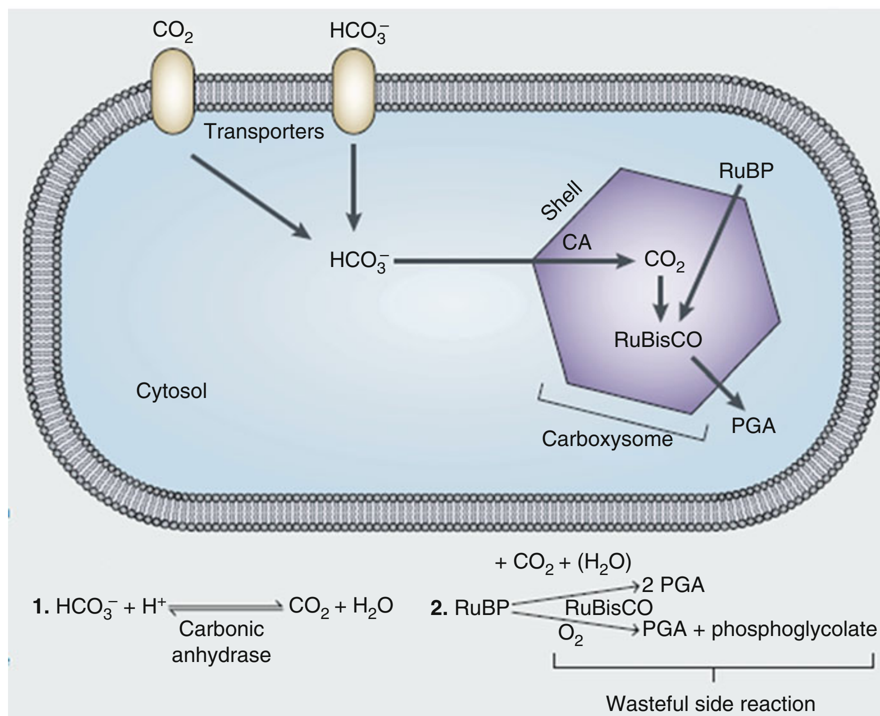
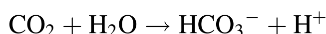


Fig. 11.5 Carbon concentration and the carboxysome (Yeates et al. 2008)

substrate of carboxysome, which is accumulated in cytosol by the help of CO_2 and HCO_3^- transporters. Here HCO_3^- is converted to CO_2 by carbonic anhydrase, then CO_2 reacts with RuBP in the presence of Rubisco enzyme to form PGA, which is later converted to glucose (Yeates et al. 2008). These transporters are located on plasma membrane and exist in both low affinity and high affinity transporter forms (Murray et al. 2003). Carbonic anhydrase is an enzyme which catalyze fast in microorganisms and plant as well. It is found in animal plants, bacteria, and archaea. There are three type of evolutionary distinct groups α, β, and γ carbonic anhydrases which show no major sequence identity or structural similarity (Ramanan et al. 2009). Carbonic anhydrase catalyzes the reversible reaction of CO_2 to form a bicarbonate anion and a proton.



$$CO_2 + H_2O \rightarrow HCO_3^- + H^+$$

11.4.8 Ribulose-1,5-Biphosphate Carboxylase/Oxygenase (Rubisco)

Rubisco is the most abundant protein on earth, carries out the catalysis of the first step in the Calvin cycle. This step is commonly considered to be the rate limiting step

of the Calvin cycle (Boyer 2006). This enzyme was discovered by Melvin Calvin at Berkeley in the 1940s (Vapaavuori 1986). Substrates such as carbon dioxide (CO_2), Ribulose 1,5 bisphosphate (RuBP), and water are required from the surrounding environment for the function of Rubisco (Ashida et al. 2003).

The substrates gather at a specific location of the active site of the enzyme Rubisco, a conformation change takes place in the enzyme and CO_2 reacts with RubBP. As a result, a highly unstable six-carbon compound intermediate is formed. The instability of this reaction intermediate, it splits into two molecules of 3-phosphoglycerate (3GP) (Vapaavuori 1986). About one sixth of the 3GP is utilized in the formation of sugars, which is considered as the output of the cycle. The active site of Rubisco is large and can accept both carbon dioxide and O_2 as substrate. The affinity of the O_2 molecule for the active site is higher and therefore its rate of binding is higher than CO_2 (Ashida et al. 2005). Different evolutionary traits in different species have been encouraged by this disorganization in the molecule (Alfreider et al. 2003).

Carboxysomes which are small vesicles that have concentrated levels of Rubisco have been developed in bacteria cells (Pichard and Campbell 1997). To maximize the exposure of CO_2 to Rubisco, CO_2 is transferred from the outside environment into the carboxysome. The oxygen is more understood in the higher plant world. There are four recognized forms of Rubisco, with forms I–III being true carboxylating Rubisco enzymes. Form IV which is also referred to as Rubisco-like protein (RLP) is found in many bacteria and archaea. RLPs which are structurally related to the true Rubiscos do not function as Rubisco enzymes, but instead catalyze different reactions in sulfur metabolism (Hanson and Tabita 2000). Form I Rubisco which is found in the cells of most autotrophic bacteria, algae, and terrestrial plants, is a hexadecamer and is made of eight large and eight small subunits (L_8S_8). Catalytic function is found only in the large subunit. It is further divided into two types, "green-like" and "red-like" and the large subunits of these two types differ in their amino acid composition (Tabita et al. 2007). The green-like Rubiscos are commonly found in the chloroplasts of terrestrial plants; green algae; cyanobacteria; and representatives of α, β, and γ proteobacteria. The red-like Rubiscos are present in most non-green algae and some representatives of α and β proteobacteria. In *Rhodobacter azoto formans*, both green-like and red-like enzymes have been found (Tabita et al. 2008).

Form II Rubisco is found only in bacteria. It has 25–30% amino acid sequence identity to form I, consists of only large subunits (Ln), from two to eight, depending on the organism, and is found in photo- and chemoautotrophs. Form III Rubisco which is only found in archaea, and its metabolic role is unclear. Form IV which is designated a Rubisco-like protein because its sequence is most closely related to Rubisco and it is not involved in the fixation of CO_2 in Calvin cycle. Table 11.1 shows the recognized presence, structure, and function of four related Rubisco enzyme forms in archaea, bacteria, and eukaryotes.

Both CO_2 (in Calvin cycle for carbon fixation) and O_2 (photorespiration) can be used by Rubisco as substrate. Some Rubisco have been selected for greater CO_2 specificity. Specificity factors are expressions of relative specificities for CO_2 versus

Table 11.1 Rubisco protein forms and their phyletic distribution

Rubisco form		Macromolecular structure	Phylogenetic occurrence	Enzymatic function
Green	Form IA	L8S8	Cyanobacteria Proteobacteria	CBB cycle
	Form IB	L8S8	Cyanobacteria, chlorophyte (green) algae Higher plants	CBB cycle
Red	Form IC	L8S8	Proteobacteria	CBB cycle
	Form ID	L8S8	Non-green algae	CBB cycle
Form II		L2	Proteobacteria Archaea Dinoflagellate algae	CBB cycle
Form III		L10	Archaea	RuPP pathway
Form IV		L2	Bacteria Archaea, including both photosynthetic and non-photosynthetic	Methionine Salvage pathway

O_2 (t = VCKO/VOKCO$_2$ where VC and VO are the maximum velocities of carboxylation and oxygenation, respectively, and KO and KCO$_2$ are the Michaelis–Menten constants for O_2 and CO_2 (Scott et al. 2007). High KCO$_2$ values and low specificity factors generally indicate that Rubisco is better adapted for a higher CO_2 and lower O_2 environment while low KCO$_2$ values and high specificity factors are found in those organisms which are adapted for CO_2 limited environments (Badger and Bek 2008). Organisms having carbon concentrating mechanisms (CCMs) can have higher KCO$_2$ values and lower specificity factors possibly because the CCM compensates for the catalytic limitation of the enzyme by increasing intracellular DIC levels (Badger and Bek 2008).

The lowest specificity factors are of Form II Rubisco and its value has ranges from 9 to 15 in organisms such as *Rhodobacter sphaeroides* and *Rhodospirillum rubrum.* These organisms are adapted for low O_2 environments (Gibson and Tabita 1977) Specificity factors of Form I Rubisco can range from 30 to 38 and it is present in proteobacteria, cyanobacteria, and green algae (Badger and Bek 2008). Specificity factors can also vary within the same type of Rubisco. For example, in proteobacteria, the specificity range of non-carboxysomal form IA is 30–35, while in cyanobacteria and some proteobacteria, carboxysome-associated form IA has a more narrow range from 33 to 37 (Badger and Bek 2008). The enzymes specificity factor are higher in organisms that are well adapted to high O_2 environments. In plants specificity factor of form IB varies from 78 to 90 and in non-green algae, specificity factors of form ID varies from 129 to 238; (Badger and Bek 2008).

There is also a great variation between different forms of Rubisco with respect to the affinities of the enzyme for CO_2. This is probably due to the great difference of CO_2 concentrations in diverse habitats. Form II Rubisco, which is generally fit for high concentrated CO_2 environments, has its KCO$_2$ values in the range of

80–247 μM (Badger and Bek 2008). Non-carboxysomal form I Rubisco has higher affinities for CO_2 and its KCO_2 values in the range of 6–138 μM (Badger and Bek 2008), while those packaged into carboxysomes, have KCO_2 values in the range of 72–250 μM; 6, 65, 69, 84, 92 (Scott et al. 2007; Badger and Bek 2008; Gibson and Tabita 1977).

11.5 Metabolic Product of CO2 Sequestration

Hydrocarbons and triacylglycerol are the major metabolic products of CO_2 sequestration in bacteria fungi, algae, higher plants, and animals. Alkane production occurs by decarboxylation of fatty aldehydes. It occurs in a three step process using different enzyme: (1) fatty acid elongase elongates hexadecanoic acid to an even-carbon number fatty acid; (2) fatty acid reductase reduces the fatty acids to aldehydes; and (3), Aldehyde decarbonylase decarbonylates the aldehydes to yield alkanes (Metzger et al. 1985; Banerjee et al. 2002). It has been reported that odd-carbon number alkanes are found in a large proportion as compared to even-carbon number alkanes in higher plants while they contain even-carbon number fatty acids as the major fatty acid components. In contrast, in some bacteria, similar levels of even and odd-carbon number alkanes have been reported, where even-carbon number fatty acids were predominant inside bacteria. As the decarbonylation pathway does not adequately explain these facts, we are forced to consider that an alternative alkane synthesis pathway may exist in the bacteria.

The fuel oils are comprised of the aliphatic alkanes (paraffins) and cycloalkanes (naphthenes), aromatics (e.g., benzene) and olefins (e.g., styrene and indene compose 10–20% and 1%, respectively, of the fuel oils (Park et al. 2005). Less than 5% of fuel oil consists of polycyclic aromatic hydrocarbons.

Triacylglycerols (TAG) are fatty acid trimesters of glycerol. There are different types of TAG depending on their fatty acid composition. Biosynthesis and accumulation of TAG occur in a group of organisms belonging to the actinomycetes, such as Streptomyces, Rhodococcus, Mycobacterium, Dietzia or Gordonia (Alvarez et al. 2002).

11.6 Biodiesel

Biodiesel is produced from renewable resources such as oil bearing plants, micro, and macro algae. It is a mixture of Fatty Acid Methyl Esters (FAMEs). Due to the shortage of the available energy resources and increase in environmental pressure on green house gases, have been forced to discover some alternative method for renewable energy sources. Biodiesel does not contribute to extra atmospheric CO_2 emissions as it is biogenic (Demirbas 2008). It reduces production of soot, sulfur, unburned hydrocarbon, and polycyclic aromatic hydrocarbon emissions as compared to petroleum diesel (Aresta et al. 2005; Demirbas 2008). It can be applied in

existing diesel engines blended with petroleum diesel. Viscosity of biodiesel has twice the viscosity of petroleum diesel, so its lubrication properties improve engine life. Biodiesel has low toxicity and it is biodegradable (Aresta et al. 2005). Burning of biodiesel emits lesser carbon monoxide, unburned hydrocarbons, and particulate emissions as compared to regular diesel fuel. Biodiesel is environment friendly hence it provides a great potential as alternative source of energy. Biodiesel is biogenic, non-toxic and fully biodegradable and it is already being tested in several countries in larger scale (e.g. 1080000 t biodiesel was developed in Germany in 2004) (Bockey and Schenck 2005). Biodiesel is manufactured by transesterification of oil or fat with methanol using either homogenous catalyst (acid and alkali) or heterogeneous catalyst (Some transition metal oxide) (Demirbas 2009). Fatty acid composition of the source (oil or fat) plays an important role in biodiesel production. Saturated fatty acids are more solidify and clog the fuel line during the winter season than unsaturated fatty acid (Demirbas 2008). The high level of unsaturated fatty acids are less viscous showing higher pour and cloud point properties which make biodiesel suitable for cold weather condition. Oleic acids are the appropriate fatty acids for the production of biodiesel.

11.7 Conclusions

The climate change and increase in carbon dioxide in the atmosphere are the major concern due to industrialization and increase in population. Many physicochemical and geological methods are reported for the removal of atmospheric carbon dioxide. Sequestration of atmospheric carbon dioxide through microbial and other microorganisms is alternative and more eco-friendly viable methods. Several bacteria such as chemoautotrophs and chemolithoautotrophs which can fix and store atmospheric CO_2. Rubisco and Carbonic anhydrase are the two enzymes which are found in the carboxysomes (a polyhedral organelle) in bacterium. Within the carboxysome, the carbonic anhydrase converts HCO_3^- into CO_2 and the Rubisco fix the captured CO_2 inside the organelle. The microbial enzymes can fix atmospheric available CO_2 into valuable products like lipids, surfactant, and polyhydroxyalkanoate components.

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Environmental Interaction and Impact on the Life Span of Stem Cells

12

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Abstract

Environment plays an indispensable role in the way an organism responds and behaves. Our body responds to different environment cues and handles them by producing various kinds of signal molecules. The behavior of an organism is in fact controlled by the individual cells which make up the organism and the behavior of the cells ultimately reflects in the behavior of the organism. The same is true for stem cells which make up the entire organism and also reside in niche of each organ. This chapter aims to summarize the existing knowledge and research conducted in the field of stem cells response to surrounding macro- and micro-environment. Micro-environment or “the niche” plays an important role in controlling the behavior of cells which is further affected by the macro-environment.

12.1 Brief History of Stem Cells

Robert Hooke in the year 1665 for the first time discovered and coined the term “cell.” This discovery further led to the development of multifaceted scientific approaches aiming to unravel the secrets of cell division, growth, and organization into complete organisms.

In 1868, Ernst Haeckel, for the first time used *Stammzelle* to describe unicellular ancestral organism as the source of evolution for all the existing multicellular organisms. In 1877, he further postulated that the fertilized egg is also a stem cell as it gives rise to all the cells of an organism. Following this in 1892, Theodor Boveri

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and Valentin Häcker conducted experiments on *Ascaris* and *Cyclops,* respectively, and postulated that the cells between the fertilized egg and committed germ cells can be considered as stem cells. It was during the nineteenth century that the pathologists Durante and Conheim for the first time hypothesized the existence of stem cells in adults as “embryonal rests” in order to include the survival of embryonic-like stem cells in adult tissues (Sell 2004). In 1981, Martin Evans and group set an important milestone by establishing an in vitro culture for non-malignant pluripotent cells derived from mouse embryos (Evans and Kaufman 1981). During the same year Gail Martin too isolated cells from the mouse embryo and named “stem cells,” since it seemed that the isolated cells could grow into nearly all cell types (Martin 1981).

The fertilized egg, which is considered to be the ultimate stem cell, is formed from the fusion of haploid germinal stem cells. In adults, the tissues are renewed by proliferation of the stem cell pool, which divide stem cells. In adults, the tissues are renewed by proliferation of the stem cell pool, which divide asymmetrically yielding an adult stem cell and a progenitor cell. The progenitor cell divides further to ultimately produce terminally differentiated cells of the tissue with a limited lifespan in the tissues which are lost by wear or through apoptosis.

The differentiating progeny of the stem cells leads to tissue renewal. It has been demonstrated that stem cells have the ability to produce progenitor cells for tissue repair and renewal which opens up vast panorama of applications in the regenerative biology. Determination or lineage commitment is a specialized function acquired by stem cells during differentiation. As the stem cells differentiate to give rise to specialized cells, with decreasing differentiation potential and finally to the terminally differentiated cells. Classic embryology considers determination as an irreversible process; once a cell is differentiated into adult, it is completely stable and not able to revert to stemness (Surani 2001). However, it has been demonstrated by the researchers that this differentiated cells can be reversed to stemness a process called “de-differentiation.” One way to do de-differentiation is through Somatic Cell Nuclear Transfer (SCNT) where a nucleus is transferred from differentiated adult cell into an enucleated oocyte which results in restoration of totipotency of the nucleus in the oocyte (Hakelien and Collas 2002; Hochedlinger and Jaenisch 2002; Surani 2001; Wilmut et al. 1997).

More recently Shinya Yamanaka has developed the concept of iPSCs (Induced Pluripotent Stem Cells). His lab has developed a methodology to reprogram the differentiated cells into stem cells which they have referred to as—induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). iPSCs have the potential to present themselves as an ideal source of stem cells source for cell-based therapy and regenerative medicine in future (Novak et al. 2010).

Embryologists and developmental biologists have known the power of stem cells for tissue development, regeneration, and renewal for many years (Sell 2004). In the recent years, a lot of research and development work has been focussed on understanding the biology of stem cells derived from different sources. Researchers are working on different aspects of stem cells including differentiation, trans-differentiation, and de-differentiation and more recently the work is initiated on iPSCs and their role in regenerative medicine. All these developments have brought

a lot of hope and opened new avenues for stem cell applications in treatment for different degenerative diseases. The field is developing at a rapid pace; we hope to see a lot of cell-based treatment modalities for degenerative diseases in near future, which is altogether set to change our current perception about a disease scenario.

12.2 Micro-environment of Stem Cell: The Niche

The word **"niche"** has different meaning in different contexts, it refers to a habitat where an organism can reside and reproduce in ecology (Scadden 2006).

Schofield for the first time in 1978 used the term "stem cell niche" describing the whole picture of stem cells survival and their interaction with the neighboring cells (Wang et al. 2016; Wang and Kingshott 2016). Stem cells produce their own matrix and milieu with the specialized micro-environment. The microenvironment of the stem cells is very complex and dynamic structure which is very much required for the self-renewal and differentiation of these cells. Due to complex structural organization of the stem cell niche it has not been characterized completely. In *Drosophila* and *Caenorhabditis elegans* the first germline stem cell (GSC) niche has been defined (De Cuevas and Matunis 2011). Several stem cell niches have been identified in different tissues including the hematopoietic stem cells (HSCs), the germline stem cells (GSCs) in ovaries and testes and MSCs in bone marrow, stem cells in the epithelia muscle satellite cells in the skeletal muscle, neural stem cells in the brain, dental pulp stem cells in the teeth, and even some cancer stem cell niches (Lane et al. 2014; Xie and Li 2007). Bone marrow is considerably the most nourishing niche for hematopoietic (HSCs) and mesenchymal stem cells (MSCs), but human HSCs can be alternatively isolated from the peripheral blood, umbilical cord, and umbilical cord blood. HSCs differentiate to form all the cells of the myeloid and lymphoid lineage (Zhang et al. 2003), whereas MSCs can differentiate into plethora of cell types including osteocytes, chondrocytes, stromal cells, cells of myotube, and connective tissue. In vitro, MSCs can be differentiated outside their lineage, which means stem cells of mesodermal origin can give rise to the cells of the endodermal or ectodermal origin, this is called trans-differentiation or stem cell plasticity (Lv et al. 2014).

One of the most important limiting steps in stem cells culture in vitro is to mimic the stem cell micro-environment in vivo. Absence of proper culture systems and protocols, normal growth of stem cells in in vitro is difficult and their differentiation potential is compromised which leads to altered state or even apoptosis (De Cuevas and Matunis 2011).

There are several structural features common between the stem cell niches of different tissues and species, however, there are difference in the functional aspects of these niches (Lane et al. 2014). Structurally the stem cell niche is highly dynamic, with multiple cell types involving complex biochemical and biophysical interactions (Ema 2012). There are niches which consist of one or few cell types which locally regulate the stem cells, whereas there are niches which consist of multiple cell types

which are involved in systemic regulation. These niches act as stem cells shelters where they maintain quiescence and self-renewal (Ema 2012).

12.3 Components of Stem Cell Niche

12.3.1 Extra-Cellular Matrix (ECM)

ECM is the essential component of the stem cell niche and plays an important role in regulating the stem cell fate (Frantz et al. 2010; Xie and Li 2007). ECM provides space and physical support to stem cells to anchor and orient (Kerever et al. 2007). Understanding and analyzing the structure and composition of ECM is important to mimic its function to make the products for synthetic cell culture. One approach to study the ECM structure and its organization is through tissue decellularization process. Decellularization eliminates the cellular components from the tissue leaving the ECM intact which can be further studied microscopically (Badylak et al. 2011; Baptista et al. 2011; Batchelder et al. 2015; Moorman et al. 2003; Sabetkish et al. 2014).

12.3.2 Surface Receptors Proteins

Anchorage dependent cells interact with their ECM by means of cell surface receptors proteins, namely integrins, selectins, and cadherins. Integrins are heterodimeric transmembrane molecules (α and β subunits) involved in cell adhesion, migration, differentiation, and even in apoptosis (Le Gall et al. 1998). The commonly expressed subunits in most human cells are β1, α5, and αV. Integrin are expressed ubiquitously in almost all the stem cells. Integrins have the ability to bind directly to the ECM proteins such as laminin, collagen, vitronectin, and fibronectin. (Assoian 1997).

Cadherins, the second type of surface receptor proteins, are regulate cell–cell interactions (Chen et al. 2013). The subunit E (epithelial) cadherin (CDH1) is best studied and is involved in adhesion of stem cell niche. Maintenance of the self-renewal potential of stem cells and retention of nondifferentiated cells in the niche are mediated via interaction with E-cadherin (Moore and Lemischka 2006).

N (neural)-cadherin (CDH2) which is another subtype of cadherins is expressed during the neuronal plate development. In the HSC niche, both HSCs and osteoblasts express N-cadherin. N-cadherin in multiple studies has also been reported to affect the self-renewal potential of HSCs in the niche (Moore and Lemischka 2006). However, few studies are emerging which counter indicate this by showing that the expression of N-cadherin in HSCs is not necessary for niche function (Kiel et al. 2009).

The satellite stem cells in the skeletal muscle niche express a third subtype of cadherin molecule M (myotubule)-cadherin (CDH15). Which is expressed at the site facing the muscle fibers (Moore and Lemischka 2006).

Apart from integrins, cadherins, selectins, and cadherins, other cell surface receptors have also been described to be crucial for stem cell niche interactions which include EGF, frizzled, Notch, TGF-ß, c-kits, gap junctions, VCAM 1, and CD44 (Chen et al. 2013)

12.3.3 Basement Membrane

Basement membrane is thin and densely organized sheet like structure consisting of self-organized ECM surrounding most of the animal tissues. Recent studies have demonstrated that basement membrane is a component of stem cell niches in many tissues including testis of *Drosophila*, mammary gland, epidermis, ovary and gut, and prostate (Hall et al. 2006; Kendrick et al. 2008; Lawson et al. 2007; O'Reilly et al. 2008; Ohlstein and Spradling 2006; Shackleton et al. 2006; Tanentzapf et al. 2007; Tumbar et al. 2004).

Laminins are the key proteins forming a component of the basement membrane proteins and consist of α6-containing integrin dimers which serve as receptors for the laminin protein. Studies have proven that stem cell properties are altered on disruption of the stem cell niche. For example, Lechler and Fuchs in 2005 demonstrated that on deleting β1 integrin or α-catenin from basal keratinocytes the orientation of the cell division plane changes resulting in altered epidermal homeostasis. Research has shown that in some mammalian tissues which include brain, intestine, mammary glands, and prostate gland, stem cells are in close contact to the basement membrane. In mammalian intestine these stem cells are localized at the base of the crypts and are in direct contact with the basement membrane (Barker et al. 2008; Haegebarth and Clevers 2009; Walker and Stappenbeck 2008). Similarly, stem cells in mammary and prostate glands reside in the basal compartment of the epithelium and interact with the underlying basement membrane directly (Lawson et al. 2007). Basement membrane-like structures in the neurogenic zones of the brain have been shown by Krever et al (2007).

12.4 Stem Cell a Target for Environmental Pollution

Extensive studies on the effect of environmental pollution on stem cells properties and accumulation of vast information has led to the development of new branch of toxicology, i.e. stem cell toxicology.

12.4.1 Stem Cell Toxicology

It is the branch of toxicology aiming to provide solution to evaluate the cellular, developmental, reproductive, and functional toxicity of the pollutant of interest using stem cells as a model system mimicking the human physiology in vitro (Faiola et al. 2015). Several pollutants such as industrial waste, pesticides, drugs, cosmetics, food

additives, radiations, smoking, and atmospheric fine particles are continuously produced in surrounding environment and adversely affects human health (Yao et al. 2016). Most of these pollutants are either slow degrading or stable in nature as a result of which they tend to accumulate in the environment. The harmful effects of these toxic chemicals on health of humans have raised a growing concern about the urgency and necessity to implement rapid, sensitive, cost-effective, novel, and high-throughput screening tests which can assess toxicity of these toxic pollutants. (Yao et al. 2016). Earlier most of the toxicity screening test for pollutants or drugs were dependent on the animal models. In 1959 Russell and Burch postulated the theory of “high fidelity fallacy,” which states that experimental animals based toxicity assays are not always translatable to human health (Russell and Burch 1959) due to inter-specific variations. For example, numerous drugs which passed the animal testing during the development process failed during the clinical trials. The principles of alternative toxicology are mainly based on in vitro studies, i.e. 3Rs (Replacement, Reduction, and Refinement) may be more important today than ever before (Gibb 2008; Krewski et al. 2008; Russell and Burch 1959).

Though animal models and in vitro cell culture systems pose several limitations including time consumption, intensive resourcing, and ethical concerns (Krewski et al. 2008), most of the toxicity screenings and research in industries and research institutes are still relying on it. Although toxicological screening systems based on in vitro human models of fast growing immortalized or cancer cell lines present a solution to this problem, these are not true representatives of the native tissue due to accumulation of mutations or altered cell functions during expansion. Toxicity screening system based on primary human cell cultures present a better option but has limitations as these cells have limited growth and proliferative potential in culture (Yao et al. 2016). Together these issues can significantly restrict the reproducibility of the tests, generation of data and its interpretation (McNeish 2004). Generally, these in vitro assays rely on the response of a single cell type and are unable to indicate correct information about the toxicological responses at the level of tissues or even whole organism where there is a heterogenous population of cells (Krewski et al. 2008).

Recently, Faiola et al. (2015) demonstrated that toxicology studies based on stem cells could be a quick, powerful, and cost-effective screening system in detecting the developmental toxicity of environmental pollutants (Jennings 2015). Human stem cell-based toxicology studies present an efficient, quick, and specific toxicity screening, establishing it as an effective model to animal experimental testing or conventional toxicity assays because it utilizes the potential of stem cells to differentiate into various cell types and tissues present in body relating it more closely to humans (Yao et al. 2016). Other advantages include less time consumption, low cost, and higher accuracy than tests using animals.

12.4.2 Somatic Stem Cell Toxicology

Apart from embryonic stem cell testing, adult stem cells also called somatic stem cells are utilized in the toxicological studies in vitro. Somatic cells represent the entire cell pool of an organism excluding the germline cells, while somatic stem cells (SSCs) are the stem cells located in the adult tissues. In the adult tissue niches, these SSCs tend to remain quiescent and maintain homeostasis at very low turnover rate. After tissue injury these cells get triggered to repair damaged tissues through multiple fold turnover of self-renewal and differentiation (Blau et al. 2015; Engstrom et al. 2015; Wang et al. 2011). With physiological aging of the body, there is a progressive disruption in the tissue homeostasis and gradual loss of the ability of SSCs to repair the damaged differentiated cells (Blau et al. 2015). With reduced repair potential of the SSCs the environmental pollutants can either cause an irreversible damage to the tissues which cannot be repaired sufficiently by proliferation and differentiation of SSCs or they can target SSCs directly, causing their exhaustion resulting their premature aging/or pathological state, including cancer and eventually death (Wilhelm Engström et al. 2015). Due to the limited differentiation potential of SSCs in comparison to ESCs, they are not utilized in embryotoxicity studies and teratogenic experiments. However, SSCs have the ability differentiate and self-renew into somatic stem cells during the periods of infancy and adolescence, thus finding their use in evaluating the detrimental effects of environmental toxins and pollutants during post-natal development of an adult organism. It was during 1980 that Robert M. Pratt and his colleagues for the first time demonstrated the application of SSCs in toxicological testing by using human embryonic palate derived for prescreening of environmental teratogens (Pratt et al. 1982). Further, Cao and colleagues first time evaluated the use of hMSCs for in vitro *screening of* cytotoxic chemicals for assessing the LD50 (Lethal Dose, 50%) values and in categorizing the hazardous status of the tested chemicals in accordance to the globally harmonized system of classification (GHS) (Scanu et al. 2011). Findings from these studies demonstrated that hMSCs could serve as precise model for mimicking the in vivo conditions in comparison to the previously established tests such as Normal Human Keratinocyte/Neutral Red Uptake methods and validated 3T3 cell test. Thus, primary tissue-derived or PSC-derived (ESC or iPSC) SSCs can be utilized in vitro for assessing the harmful effects of pollutants on the development of infants and adolescents into adults. SSC-based injury or disease models can also be used in specific applications where the toxic effects of chemical during tissue repair following injury or degenerative diseases are to be assessed. They can also be used in determining the effects of pollutants on stem cell aging and exhaustion.

Akhavan and group (Akhavan et al. 2012) evaluated the toxicity of graphene based materials on SSCs. They demonstrated that reduced graphene oxide nanoplatelets at low concentrations (0.1 mg/mL) exerted genotoxic effects on hMSCs as a result of chromosomal aberrations and DNA fragmentation. Later, Strong and colleagues studied the toxic effect of endocrine-disrupting chemical dichlorodiphenyltrichloroethane (DDT) on SSCs. The groups treated hMSCs with DDT and revealed significant modifications in the stem cell properties including

proliferation, self-renewal, differentiation (adipogenic and osteogenic lineages), and in gene expression, which could partly justify the homeostatic imbalance caused and increased risk of cancer occurrence among the individuals exposed to the chemical (Amy et al. 2014).

In another study, Tamm and his group demonstrated that primary human embryonic cortical stem cells and C17.2, a neural stem cell line expressed high sensitivity on exposure to methylmercury (MeHg) at low levels. Their findings indicated the effects of MeHg on survival, proliferation and differentiation of these cells, offers new avenues for studying the biological outcomes of low level exposure of this chemical using highly sensitive and reliable in vitro models (Tamm et al. 2006). In 2009, Buzanska and group (Buzanska et al. 2009) established an umbilical cord blood derived neural stem cell line and used it for testing the neural development based toxicity by analyzing parameters such as cellular proliferation, neuronal and glial differentiation, and apoptosis.

It can be widely accepted that, by employing these stem cell model for extensive in vitro testing for identifying the significant toxicity mechanisms at the cellular and molecular levels on human biology, we would be able to eliminate the requirement of animal testing and would be able to cater decent environment-friendly and healthy decision-making in future.

12.5 Stem Cells, Environment, and Cancer Risk

One of the key factors in the battle against cancer is understanding and determining the underlying cause for this devastating disease. The potential target of cancer and site of occurrence of cancer is usually defined with the help of environmental and genetic factors (Zhao 2015). Recently, a study by Wu and group has confirmed the role of environmental factors including ionizing radiations, ultraviolet radiations, and carcinogens in causing cancer (Wu et al. 2015)

According to their calculations, intrinsic (random errors in DNA replication) risk can be effectively determined by the lower bound risk accounting for total stem cell divisions and the internal processes are not adequate to account for the cancer risks observed. Also, there have been overwhelming scientific evidences which establish genetic and environmental factors as critical players in the development of cancer (Zhao 2015).

12.6 Embryonic Stem Cell Test

Embryonic Stem Cell Test uses mouse embryonic stem cells (mESCs) as model system to (Seiler et al. 2004; Seiler and Spielmann 2011) test the embryotoxicity of pharmaceutical compounds in vitro. This technique was pioneered by Horst Spielmann in 1991. During the initial stages of development of the assay it was not precise with poor rate of prediction. It was only during 1995–2004 that the European Centre for the Validation of Alternative Methods (ECVAM) nominated,

described, and approved (Genschow et al. 2002; Spielmann et al. 2008) EST as the in vitro assay for embryotoxicity. Since then, this assay has been utilized for drug screening (Paquette et al. 2008; Whitlow et al. 2007) to study the toxicity of a panels of related compounds on development (de Jong et al. 2009) and even for assessing embryotoxicity of nanomaterials (Di Guglielmo et al. 2010). Establishment of EST is considered a milestone in the history of stem cell toxicology testing because of its advantage over in vitro and pre-clinical tests (Genschow et al. 2002).

Even though considered as a very effective assay for toxicology testing, EST, did have several weaknesses such as for assessing myocardial differentiation, microscopic observation was the sole means for studying the beating areas whereas no metabolic tests for analysis were not available (Spielmann et al. 2006). Further, limitation in the EST prediction model was highlighted by another study which was sponsored by a ECVAM and ReProTect where EST based analysis was able to classify only 15% of previously untested compounds correctly (Marx-Stoelting 2009).

With the development and technological advancements in the field of biotechnology accompanied with the emergence of new molecular techniques and tools, modifications in the EST assay were proposed which could overcome the existing limitations. For evaluating beating cardiomyocytes, the visual microscopic evaluation was replaced by highly sensitive and specific techniques such as fluorescence activated cell sorting (FACS) and reverse transcription quantitative PCR (RT–qPCR) providing more quantitative molecular endpoints analysis in terms of gene and protein expression of myocardial markers (Bigot et al. 1999; Buesen et al. 2009; Pellizzer et al. 2004b; Riebeling et al. 2011; Seiler et al. 2004; Seiler 2006; Zur Nieden et al. 2001).

Further, to assess the toxicity of pollutants on various tissue and organs tests were developed on the basis of the potential of stem cells to differentiate into multiple lineages including pancreatic, neuronal, osteogenic, and skeletal muscle lineages (Schmidt et al. 2001; Mori and Hara 2013; Pellizzer et al. 2004a, b; Rolletschek et al. 2004). Based on the new advancements, in 2004 a new EST subtype referred as molecular multiple-endpoint EST developed, which not only included traditional cardiomyocyte differentiation as an assay parameter but also incorporated quantitative analysis using RT-qPCR and multilineage stem cell differentiation-based assessment as additions (Zur Nieden et al. 2004).

Above described ESTs used differentiated fibroblasts (3T3 cell line) and ESCs as assay model. For simplifying and improving the reproducibility of EST based testing, development of advanced procedures relying exclusively on ESCs were targeted. High-throughput toxicological analysis techniques such as miRNA and whole genome profiling using techniques like microarrays and mass spectrometry were incorporated.

Moreover, the advantage of ESCs over cancerous and other cell types is that they can be used in the development of toxicity assay. For instance, ESCs have ability to grow into three-dimensional cell aggregates the so-called organoids. These organoids or embryoid bodies (EBs) can be considered as miniature organs as

these are useful in mimicking the early stages of embryonic development occurring in vivo (Wobus and Loser 2011) (Liu et al. 2013; Mori and Hara 2013).

As the results of toxicological analysis performed using mESCs could not be applied to humans directly due to inter-generic variations, Cezar and group introduced use human embryonic stem cells (hESCs) as model in toxicology testing (Gabriela et al. 2007) to overcome the drawbacks associated with mESCs. They performed small metabolite-based profiling using mass spectrometry and demonstrated that hESCs and hESC-derived cell types (for example, neural precursors) exposed to pollutants could be helpful in elucidating the molecular mechanisms associated with toxicity. On performing metabolomic profiling using hESC model, biomarkers for developmental toxicity were identified and it was demonstrated that the teratogenicity was correctly predicted for 88% of drugs and 83% of environmental toxicants.

Further, fibroblasts derived from hESCs were utilized for in vitro toxicology screening and it was shown that hESCs derived fibroblasts displayed more sensitive dose response to mitomycin C treatment in comparison to other in vitro models such as human lung fibroblast L929 cells (Cao et al. 2008). Several studies utilized hESCs for specifically assessing toxic effects of drugs or compounds on neural differentiation only.

12.7 Artificial Niche

Harrison et al. in 1907 reported the first cell culture. Since then, with the advent of new technology and scientific advancements there has been a tremendous improvement in the techniques of cell culture. These advancement culture methods include mouse ESCs culture established by Evans (Evans and Kaufman 1981), culture of human ESC by Thomson (Thomson et al. 1998), mouse iPSC culture establishment of by Shinya Yamanaka (Takahashi and Yamanaka 2006), and recent establishment of human iPSC culture by Yamanaka and Thomson (Takahashi et al. 2007; Yu et al. 2007).

With establishment of new and advanced culture techniques it has been accepted that culture protocols require standardization depending upon the cell type. For example, in case of umbilical cord blood and bone marrow derived MSCs culture, tissue culture plate surface (TCPS) using a specific cell culture medium is sufficient for expansion of MSCs. The TCPS properties allow for the adsorption of the serum proteins present in medium (e.g. fibronectin, vitronectin, etc.) on the TCPS thereby supporting cellular adherence. Alternatively, while using human ESCs, iPSC and several other sensitive cultures including neural stem cells, an ECM protein pre-coated surface is required.

Although TCPS with various surface treatments are commercially available which provide charged surfaces and low cellular attachment, these surfaces do not replicate the true physical and structural cues which are important for determining the correct cell fate. More complicated culture protocols are required involving early progenitor cells (e.g. ESCs or GSCs) where stem cell niche interactions are

important for regulating their fate in vitro. Though stem cells can be conveniently cultured and expanded on 2D synthetic surfaces, there is a gradual loss in their ability to self-renew, proliferate, and differentiate into tissue specific cells. Hence, to mimic the stem cell niches in vitro the minimum requirement is a complex micro-environment including a cell specific surface with topographical distribution of physical cues to support cell attachment and movement such as matrigel for 3D organoid culture or flexible surfaces for differentiation into contractile muscle cells or even complex cell culture approaches like 3D culture, co-culture, dynamic cultures, physical stimulation, or multiple combinations of the described approaches. It is clear that the existing tools and strategies for culture are limited and require improvement for the progress of our understanding about the cell functions on different surface and strategies for controlled differentiation into clinically relevant cell types. The key factors regulating cells fate in vitro are medium composition, surface chemistry, and substrate surface topography. A plethora of reports have been published describing the standardization of the above mentioned factors to effectively mimic stem cell niches *in vitro* (Ding et al. 2016; Schuldiner et al. 2000).

Recent development of recombinant proteins have proven to be an improvement over the currently used mouse embryonic fibroblasts (MEF) supported cultures (e.g., M of human PSCs. Being sensitive, PSCs require complex medium with additional growth factors and ECM to support their self-renewal in vitro compared to other more differentiated cells. Although several serum free media compositions have been developed and available commercially in conjunction with modified TCPS with ECM or cell binding coatings but they still might not be able to maintain the native state of these PSCs (Theunissen et al. 2014).

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Pharmaceuticals: An Emerging Problem of Environment and Its Removal Through Biodegradation

13

Kritika Sharma and Garima Kaushik

Abstract

Human pharmaceuticals are consumed in high quantities worldwide and this amount will keep on increasing because of improving health care systems and longer life expectations. Due to their extensive use and release in the environment, these compounds have been found to cause harmful impacts in various environmental matrices. Pharmaceuticals can be exposed into the environment by various routes like manufacturing units of pharmaceuticals and effluents from the hospitals, irrigation to agricultural lands (e.g., biosolids and reuse of water), human and animal use, etc. The fate of pharmaceuticals depends on various physicochemical and environmental factors. Consequently, they enter surface water systems where they may pose effects on aquatic life and finally may enter the human water cycle. So, the removal of these compounds has become an urgent need of time. Conventional wastewater treatment plants are not adequate to degrade all type of pharmaceutical compounds. Hence, cost-effective and environment friendly techniques are required for eliminating these compounds from the ecosystem. Biodegradation is one such reliable method for removing harmful chemicals compounds without disturbing the environment. This work reviews the presence, fate, and microbial degradation of drugs and also suggests viable options for their removal.

13.1 Introduction

The use of chemical substances for agricultural and other household activities is gradually increasing. They are accountable to expend various emerging compounds in the environment. Various drugs are extensively used for human and veterinary

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Fig. 13.1 Industrial discharge of pharmaceuticals and its impact on aquatic organisms

purpose and constantly introduced into ecosystems which exhibit "pseudo- persistence" (Radke et al. 2010). Stumm-Zollinger and Fair in 1965 first observed the harmful effects of pharmaceutical compounds in municipal wastewater and the occurrence of pharmaceutical residuals in environment was articulated by Tabak and Bunch in 1970 (Jones et al. 2005). They are categorized as recalcitrant bioaccumulative compounds and are thus regarded as hazardous chemicals (Homem and Santos 2011). A large amount of pharmaceuticals is excreted in the aquatic and terrestrial environment in various ways. Generally, hospitals, industrial wastewater, uncontrolled drug disposal are an important source of aquatic pollution and loss of aquatic organisms due to the presence of a higher concentration of pharmaceuticals (Patel et al. 2019) as shown in Fig. 13.1.

Pharmaceuticals production industries are rapidly developing and extensively generate products used by human and animals (Qian et al. 2015). India ranks third in the whole world in terms of the number of pharmaceutical industry and fourteenth in terms of cost. Now various pharmaceutical companies are rapidly growing in selected states in India and discharge their effluents into water bodies (Chander et al. 2014). Potential risks associated with the discharge of pharmaceutical compounds into aquatic environment have become major issues for environmental regulators and pharmaceutical industries (Jørgensen and Halling-Sorensen 2000). Since last two decades pharmaceutical wastes in soil and surface water have been widely investigated (Sui et al. 2015). For this various analytical techniques were employed to illustrate the presence of complex mixture of pharmaceutical compounds in effluents and more specifically the fate of those compounds which changes their structure time to time and converted into their metabolites to form new compounds (Riva et al. 2015). These pharmaceutical compounds may cause various harmful impacts. For example, pathogen resistance can be increased due to the presence of pharmaceutical compounds in environment (Finley et al. 2013). Resultantly they induce genotoxic and cytotoxic effects on the living organisms. Steroids are endocrine disrupters which can inhibit the development, reproduction, and other hormonal misbalances that control various processes of species (Porte et al. 2006). Neurotransmitter regulation is transformed by some pharmaceuticals and they interrupt the central and peripheral nervous system in vertebrates and invertebrates. Psychiatric drugs are the most common toxic drugs to aquatic organisms (Santos et al. 2010). The environmental concentration of pharmaceuticals even at a range of ng/L to g/L can cause direct harmful impacts on environment and humans (Gros et al. 2010). Parameters which are used to determine the environmental exposure concentrations of pharmaceuticals include degree of usage, the extent of release into the environment, and its environmental persistence. Persistence of

pharmaceuticals in environment depends on various kinds of features such as inherent physicochemical properties of pharmaceuticals, different environmental factors like pH, light, temperature, etc. and their presence and biodegradation potential of microorganisms via metabolic or co-metabolic pathways (Onesios et al. 2009).

In recent times, terrestrial and aquatic ecosystems have brought about significant and regulatory concern due to extensive detection of pharmaceuticals in the environment (Zuccato et al. 2010; Cardoso et al. 2014). Predominantly pharmaceuticals have been detected in WWTP effluent, surface waters like river stream, and less commonly in drinking water and groundwater (Rivas et al. 2012). Wastewater treatment processes in WWTPs are not so efficient to remove pharmaceuticals; thus, the effluent from wastewater treatment plant becomes major source point for aquatic exposure to pharmaceuticals (Zuccato et al. 2005). Sewage sludge in the form of biosolids and manure is used as a crop fertilizer, and for irrigation purpose reclaimed water containing human and veterinarian pharmaceuticals is used which penetrates to agricultural soil (Topp et al. 2008). Thus pharmaceuticals can leach into groundwater through soil (Fent et al. 2006). They are also detected in coastal seawater (Roberts and Thomas 2006). Over the past many decades' awareness and concern have grown toward this issue. Pharmaceutical compounds in the wastewater treatment plants are controlled by various important mechanisms comprising sorption, desorption, aerobic and anaerobic biotransformation, biotic and abiotic degradation, and volatilization. Though some advanced methods like biodegradation using capable microbes can be useful in the elimination of pharmaceutical compounds. The use of microorganisms in biodegradation of pharmaceutical compounds and other xenobiotics has been found as an eco-friendly and cost-efficient tool for wastewater treatment (Tran et al. 2013). Various microorganisms like bacteria, fungi, and algae have been proved better degraders for contaminants in wastewater (Ding et al. 2017; Castellet-Rovira et al. 2018; Woźniak-Karczewska et al. 2018). Microorganisms have the capability to use target compounds as their carbon, nutrients, and energy sources to degrade them or change into less harmful compounds. Various researches have studied bacterial degradation and biotransformation of pharmaceutical compounds intensively in the past few years (Gauthier et al. 2010; Sharma et al. 2017). In this chapter we will study these biodegradation methods in detail.

13.2 Occurrence of Pharmaceuticals in the Environment

Pharmaceutical compounds enter in an environment by various routes as shown in Fig. 13.2.

The major factors for determination of environmental exposure of various concentrations of pharmaceuticals include the quantity of consumption, extent of discharge in environment, and degree of persistence in environment. Pathways by which the pharmaceutical compounds enter to environment can be different depending on the consumption of pharmaceuticals. Pharmaceutical industries use

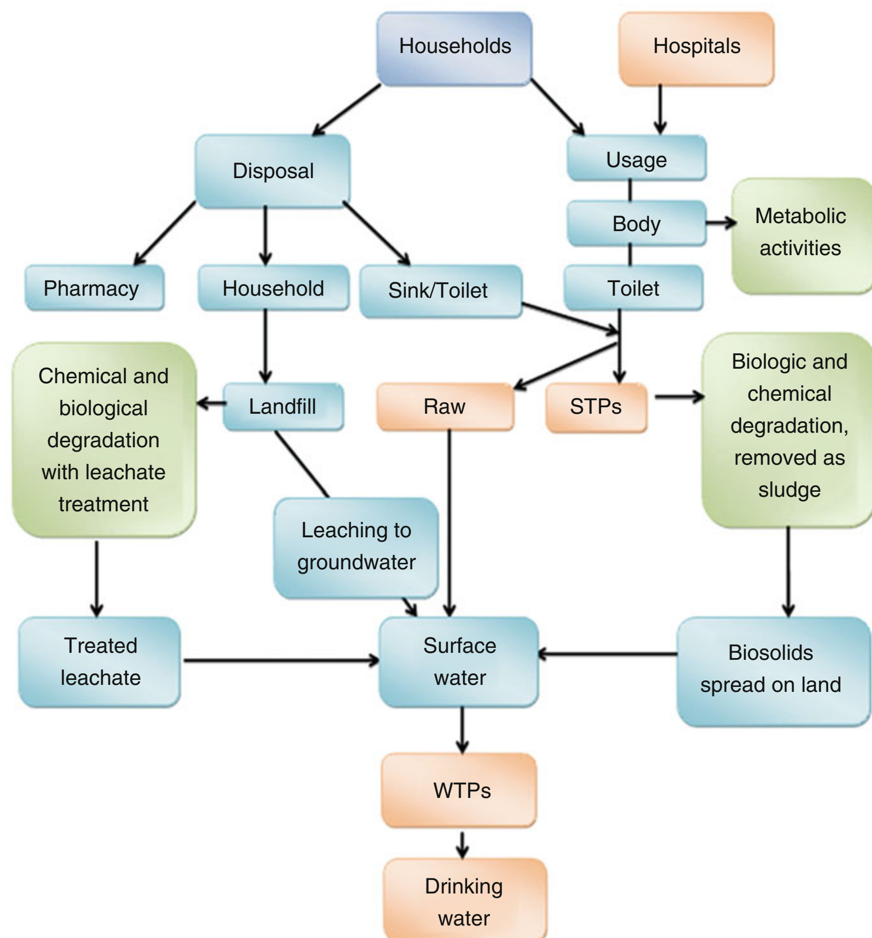

Fig. 13.2 Fate and transport of pharmaceuticals in the environment (modified from Heberer 2002)

the accounted "active pharmaceutical ingredients" to depict these products as pharmacologically active, degradation resistant, extremely enduring in aqueous media and have the potential to create adverse impact on aqueous flora and fauna as well as on human health (Rivera-Utrilla et al. 2013).

The pollution raised by pharmaceuticals in surface water (Agunbiade and Moodley 2016), groundwater (Paíga and Delerue-Matos 2016), WWTPs (Kostich et al. 2014) has been reported by many scientists all over the world as emerging environmental obstruction. During the last few years' Indian pharmaceutical industries have recorded a fast increment. Production under pharmaceutical industries comprises raw material, various kind of medicines like anti-inflammatory, antibiotics, hormones, cosmetics, and personal care products that produce harmful chemical containing effluent during their manufacturing (Dixit and Parmar 2013).

Various researchers have shown long term impacts of pharmaceuticals in environment (Mompelat et al. 2009; Boxall et al. 2012). Pharmaceuticals and their metabolites even in trace amounts have been detected in aquatic (drinking water, groundwater, and surface water) and terrestrial environment (Vieno et al. 2007; Vulliet et al. 2011). A remarkable portion of pharmaceuticals is released in various environmental sectors as parent, secondary metabolites and conjugate forms (Zhou et al. 2012). Increasing use of pharmaceutical compounds and their frequent discharge in the environment is making stressful conditions for less efficient wastewater treatment systems. In addition, the presence of pharmaceutical compounds can also pose danger by reuse of treated wastewater (Behera et al. 2011). Due to partial removal potential of WWTPs and uncontrolled discharge, assorted range of various antibiotics have been found in surface water, groundwater, sediments, soil, and animal manure which trigger off the detrimental effects in non-targeted organisms (Song et al. 2010; Leung et al. 2013).

13.2.1 Wastewater Treatment Plants (WWTPs)

Environmental occurrence of pharmaceuticals genuinely depends on the production rate, the dosage, their usage, environmental persistence, metabolic activities along with the removal efficiency of wastewater treatment plants (Jelic et al. 2011). Generally, wastewater containing pharmaceutical compounds generates from numerous sources like hospitals, health care centers, veterinary clinics, research laboratories, households, and pharmaceutical manufacturing units and ultimately reaches to WWTPs before discharging in the aquatic environment. Though the removal of pharmaceuticals in WWTPs is found improper in various studies, because of type of treatment processes, their compound specific properties, WWTPs related factors, amount and patterns of pollutants, etc. (Gulkowska et al. 2008).

Mainly WWTPs consist of a primary kind of treatment system as physicochemical treatment and secondary treatment which comprises biological treatment initiated by activated sludge (Douziech et al. 2018). These conventional treatment plants have inadequate potential to eliminate pharmaceuticals from wastewater. Various refractory compounds do not degrade even by microorganisms and may reduce the microbial activity and thus they accumulate in the food chain. Though further research is requisite on the improvement of efficiency of traditional WWTPs (Rivera-Utrilla et al. 2013).

13.2.2 Groundwater

Groundwater is the key contributor in many streams and rivers and consequently it is very important for rivers and wetland habitats flora and fauna. In the past few years the threat of emerging pollutants in groundwater contamination is increased due to climate change, unawareness, increasing demands of pharmaceuticals, and

urbanization that negatively influence the natural aqueous ecosystem because of their transportation from surface water to groundwater (Heberer 2002; Lapworth et al. 2012).

Groundwater pollution may be initiated from various potential sources comprising septic tanks, sewer leakage, runoff, contaminated landfills, solid waste, gasoline spills, leaching of contaminants from contaminated water bodies and rivers, seawater intrusion, etc. Moreover, due to the slow flow rate of groundwater and long-distance make pollutants difficult to be discharged from groundwater. Therefore, the emerging contaminants can stuck in the layer of groundwater (Lin et al. 2015). The long term persistence of antibiotics even at low levels may support the abundance of antibiotic resistant bacteria in the river base flows (Watkinson et al. 2009). In the past few decades, advanced analytical techniques made it feasible to determine the trace level concentration of PPCPs in the groundwater. These studies determined the presence of pharmaceuticals in groundwater at noticeable concentrations and their distribution in groundwater resources (Fram and Belitz 2011).

13.2.3 Surface Water

Recently, the occurrence of pharmaceuticals in surface waters is well reported in European and North American countries (Weigel et al. 2004; Nikolaou et al. 2007). Marine water bodies can act as container of antibiotic residues (Zhang et al. 2013). Huge amount of antibiotics travels to coastal waters from land through riverine inputs (Zheng et al. 2012). Furthermore, after discharging of pharmaceuticals in aquatic environment, they may reach to aquifers and change the groundwater quality, which simultaneously combined with surface water and frequently act as a critical source of drinking water supply (Mastroianni et al. 2016). In the water sample collected from the Eastern Mediterranean Sea, the presence and spatial distribution of 158 pharmaceuticals and illicit drugs were analyzed as this area from treated wastewater of Athens (Alygizakis et al. 2016). Reclaimed water is recently used for agricultural irrigation, especially in arid and semi-arid regions as sustainable source of water. Consequently, attention has been raised for potential threat of these types of practices as the reclaimed water may contain different kind of pollutants like pharmaceuticals and personal care products (Qin et al. 2015).

13.2.4 Soils and Sediments

Pharmaceuticals have been found in trace amounts in soil irrigated with reclaimed water (Kinney et al. 2006). Frequent concoction having bioaccumulation properties may cause the consequences of bio-uptake and pharmaceutical contamination in agricultural soils (Qin et al. 2015). Sorption of pharmaceuticals may occur from sewage sludge during the sewage treatment process and from where it enters the soil by sludge and treated wastewater utilization in agricultural farms as fertilizers and irrigation, respectively (Oppel et al. 2004; Ternes et al. 2007) and ultimately enters

the food chain. Various examples have been observed which support the existence of pharmaceuticals in soil which causes the harmful impact on non-target organisms in different ways such as alteration in sex ratio of higher organisms, specific changes in biogeochemical cycles, changes in plant growth, failure of larvae to molt, and anatomical deformation of various organisms (Pounds et al. 2008; Parolini and Binelli 2014). Higher concentrations of veterinary antibiotics in swine and chicken manure indicated immense usage of veterinary antibiotics in swine and chicken husbandry (Hou et al. 2015).

13.3 Environmental Fate of Pharmaceuticals

Study of the fate of pharmaceutical substances with their released amount is essential. Distribution and fate of pharmaceuticals depend on the range of various factors like physicochemical properties, their distribution in soils and sediments, and degradation in aquatic and terrestrial environment. Climate and type of soil also influence the fate and distribution of drugs (Boxall et al. 2003). Generally, drugs rarely hydrolyzed. Most of the pharmaceuticals absorb inadequately and are also not absolutely metabolized in animals and human beings. Large amount of the intake dosage of pharmaceuticals are released via excreta within few hours of its consumption as parent compound or in metabolite form (Zhang et al. 2014a, b). Usually, pharmaceutical compounds reach the environment in the form of mixtures which are very dangerous to living beings (Stackelberg et al. 2004). During wastewater treatment process some pharmaceuticals are not completely degraded and get released in treated effluents and solid waste. This solid waste is used as fertilizers on agriculture lands.

Apart from ecological threats, humans are also concerned with its menace. A large number and various kinds of antibiotics along with their metabolites remain in reclaimed wastewater varying their composition and concentration (Petrie et al. 2015). When reclaimed wastewater (RWW) is irrigated to the crops it got exposed in agricultural land with antibiotics as well as antibiotic resistant bacteria. There they multiply in the soil and may cause severe health impacts to human health. On the other hand, when soil amendment is done with drug residue containing manure and biosolids and irrigation is done with RWW, then their residue may undergo with sorption, desorption, biotic and abiotic transformation process. Thus, they harshly affect the soil microbiota. Plants uptake the pharmaceutical residues and bioaccumulate in plant tissues and later enter into the food web (Christou et al. 2017).

The drugs may undergo direct and indirect photolysis and it is the main approach of abiotic transformation in water streams. In direct photolysis there is direct absorption of sunlight whereas in indirect photolysis natural photosensitizers take part (Nikolaou et al. 2007). The dissipation process of pharmaceuticals in the environment can be inhibited by various procedures. For this, key processes consist of aerobic and anaerobic biodegradation and abiotic transformation; for instance, degradation by UV-light, hydrolysis, and sorption. According to the type of drug and

climate of that place, the most effective process can be detected. But among all, aerobic and anaerobic processes are most significant for the elimination of pharmaceutical from the dissolved phase (Tambosi et al. 2010).

In wastewater treatment process biodegradation and adsorption are two important processes.

Usually, pharmaceuticals in the acidic form in sludge are removed by adsorption. For the removal of acidic pharmaceuticals to sludge, adsorption is suggested as secondary treatment (Urase and Kikuta 2005). When pharmaceuticals are present majorly in dissolved phase, then most important elimination process of wastewater treatment is biodegradation. Biodegradation process can be aerobic and anaerobic. While pharmaceuticals are found in dissolved form, the anaerobic degradation is recommended as most significant removal method of pharmaceuticals. Moreover, as the hydraulic retention time increases, the biological decomposition of pharmaceuticals also increases (Yonetani et al. 2017).

13.4 Biodegradation of Pharmaceuticals

According to US Environmental protection agency "Biodegradation is a process by which microbial organisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment." Biodegradability indicates the transformation of organic compound by microorganisms (bacteria, fungi, yeast, and algae) to simple form of molecules during biological activities. Biodegradability signifies the potential of microbes to mineralize the organic contaminants. The final products of the degradation process remain in the form of water and carbon dioxide (Murphy and Morrison 2002).

Indigenous microbial communities of soil and aquatic environment are the "regulators" as they provide ecosystem services like monitoring the quality of ecosystem, fate of pollution released into the environment, and degradation of pollutants (Reed and Martiny 2007). Microbes in the ecosystem have the quality of self-modification and growth as they can degrade the pollutants by their metabolic and co-metabolic activities. Biodegradation is deemed as the most significant process for the elimination of pharmaceuticals from the environment (Rodarte-Morales et al. 2012). Processes of biodegradation acquired by microbes are relatively restricted due to the presence of xenobiotic compounds. Pharmaceuticals possess kinds of elements which can extensively alter physicochemical properties of the environmental and biochemical performance of the organisms. Recovery from the contamination is feasible if the toxicity of molecules does not hinder the microbial activity (Lahti and Oikari 2011). Biodegradation of recalcitrant compounds involves the augmentation of vigorous microbial culture along with the determination of their metabolic and kinetic parameters. Biodegradation of pharmaceuticals is considered as eco-friendly, cost-effective, and sustainable method and has shown the potential to eradicate pharmaceutical residues by degrading them into nontoxic end products (Hasan et al. 2011). Various biodegradation studies related to the removal of pharmaceuticals of different range in various environmental sectors have been

reported (Bessa et al. 2017; Sharma et al. 2020). *Streptomyces* spp. demonstrated the biotransformation of Clofibric acid under submerged conditions due to co-metabolic pathway.

13.4.1 Aerobic Biodegradation

According to the National Research Council "Aerobic biodegradation is the degradation of compounds by microorganisms in the presence of oxygen". In aerobic biodegradation for the transformation of organic components to simpler products, the microbe converts oxygen to water. Oxygen acts as an electron acceptor under the aerobic conditions. In aerobic biodegradation process, the pollutant degradation rate is habitually inhibited by the presence of oxygen which is provided by the atmospheric diffusion or dissolved in and carried by water. The atmospheric diffusion of oxygen into water and soil slows down and water solubility of oxygen is also low. Recently, emerging contaminants such as pharmaceuticals and personal care products have been primarily focused on aerobic biodegradation. Aerobic degradation is more suitable method rather than anaerobic degradation (Ghattas et al. 2017). To examine the sorption and degradation of bisphenol A at g/L range by aerobic activated sludge lab-scale batch experiments were conducted. BPA elimination in the aerobic activated sludge system was determined by sorption on activated sludge and biodegradation (Zhao et al. 2008). Aerobic degradation of tylosin was performed in water and soil to examine its environmental fate. It was degraded by a half-life of 200 days in light and water (Hu and Coats 2007). Complete elimination of naproxen was obtained within 14 days by addition of external carbon source under aerobic conditions (Lahti and Oikari 2011). Construction of treatment wetland for wastewater treatment, the aerobic conditions of water and soil column plays a major role to enhance the treatment effectiveness for pharmaceuticals. As compared to anaerobic conditions the degradation of specific compounds was higher in aerobic conditions. Under aerobic conditions ibuprofen, DEET and gemfibrozil usually had the half-life about 20 days (Conkle et al. 2012). Deng et al. (2016) reported that for the degradation of sulfadiazine, two aerobic bacterial strains D2 and D4 of genus *Arthrobacter* were isolated from sulfadiazine containing activated sludge. For strain D2 half-life time for the degradation was 11.3 h and for D4 it was 46.4 h. For aerobic degradation of sulfamethoxazole activated sludge was used as sole carbon and nitrogen source and microbes utilized it for their growth (Müller et al. 2013). Thus aerobic biodegradation has been proved as convenient and sustainable method to degrade pharmaceutical compounds in environmental matrices.

13.4.2 Anaerobic Biodegradation

The process for anaerobic digestion is usually employed to treat settled and semi-liquid sludge. Anaerobic biodegradation is regarded as fermentation process having four steps such as hydrolysis, acidogenesis, acetogenesis, and methanogenesis which

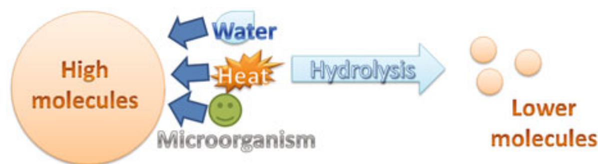

Fig. 13.3 Process of hydrolysis for the degradation of pharmaceutical compounds

is usually carried out by hydrolyzing and methanogenic bacteria (Ji et al. 2013). *Clostridia*, *β-Proteobacteria*, *Methanomicrobia*, *Thermoplasmata*, *Methanobacteria* are foremost classes of bacteria that are widely used in anaerobic process (Shi et al. 2015, 2017). Several recalcitrant contaminants are not biodegradable under aerobic conditions but they are prone to degrade under anaerobic conditions (Ghattas et al. 2017). During the depletion of oxygen in aerobic process, nitrate can be utilized as optional electron acceptor by denitrifying bacteria which are generally facultative anaerobes. Reduction of manganese and iron occurs under redox potentials. Contaminants, like benzene, toluene, ethylbenzene and xylene (BTEX), and carbon tetrachloride are degradable under these conditions (Boopathy 2002; Laban et al. 2010).

Sulfate reducing microbes use sulfate anions as alternate electron acceptor for the degradation of chlorinated compounds and polycyclic aromatic substances (Boopathy 2002; Bergmann et al. 2011). Methanogenesis comprises of biological degradation of organic contaminants in the deficiency of inorganic electron acceptor except carbon dioxide, such as halogenated aromatic compounds and triclosan (Liang et al. 2011; Veetil et al. 2012). Acetogenic bacteria oxidize the reduced organic fermentation products to substrates for methanogenesis. Halo respiring bacteria which are independent growing microorganisms and inorganic electron acceptor are capable to remove the halogenated organic contaminants (Holliger et al. 1998). Thermal hydrolysis is employed successfully for commercial uses to rapidly breakdown cell walls and to solubilize the organic matters as shown in Fig. 13.3 (Xue et al. 2015).

Shi et al. (2017) reported some advanced technologies having potential to efficiently remove pharmaceutical waste such as anaerobic membrane bioreactor (AnMBR), upflow anaerobic sludge blanket (UASB), anaerobic sequencing batch reactor (AnSBR), moving bed biofilm reactor (MBBR), etc. AnMBR is collectively anaerobic process and the membrane filtration to generate the effluent without any solids by absolute biomass retention (Ng et al. 2014). Additionally, it has a major role in energy production having methane formation capability from the large portion of organic contaminants present in wastewater (Skouteris et al. 2012). For industrial and domestic wastewater treatment Upflow anaerobic sludge blanket (UASB) is most extensively used anaerobic system owing to inexpensive, durability, high biomass concentration, diverse range of microbes (Aida et al. 2015). It is used for the pre-treatment of pharmaceutical wastewater as well as bench scale treatment of pharmaceutical wastewater. The anaerobic sequencing batch reactor (AnSBR) is five stage activated sludge process, i.e., filling, reaction, decantation, and idle in the absence of oxygen and in dark conditions (Shi et al. 2017).

13.5 Role of Different Microbes in Degradation of Pharmaceuticals

Persistence of pharmaceuticals in the environment depends on diverse factors such as their physicochemical properties, environmental factors like pH, temperature, light and the most significant is existence and metabolic or co-metabolic activities of microorganisms to degrade the pharmaceuticals (Onesios et al. 2009). Dependency of microbial degradation is shown in Fig. 13.4.

The metabolic pathways of biodegradation refer to the utilization of energy source like carbon, nitrogen, minerals, and other nutrients. Casual decomposition of a contaminant by an enzyme or other cofactor which is formed through microbial metabolism of another compound is termed as co-metabolism (Horvath 1972). A simple degradation process of pharmaceuticals by microbes is depicted in Fig. 13.5.

For example, the degradation pathway of ibuprofen drug by bacteria *Micrococcus yunnanensis* is shown below in Fig. 13.6.

Thus, microorganisms play a very crucial role in retaining the ecosystem functioning. In aqueous and terrestrial environment microbial communities play a major role in controlling the ecosystem quality and regulation of outcome of pollution released into the ecosystem (De Groot et al. 2002). Degradation of 0.1–1.0 g/L

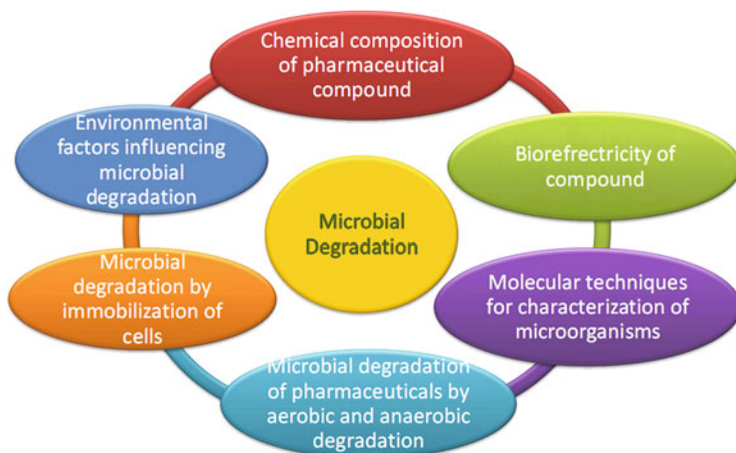

Fig. 13.4 Factors affecting the microbial degradation (modified from Varjani and Upasani 2017)

Fig. 13.5 Common process of microbial degradation of pharmaceuticals (modified from Salama et al. 2017)

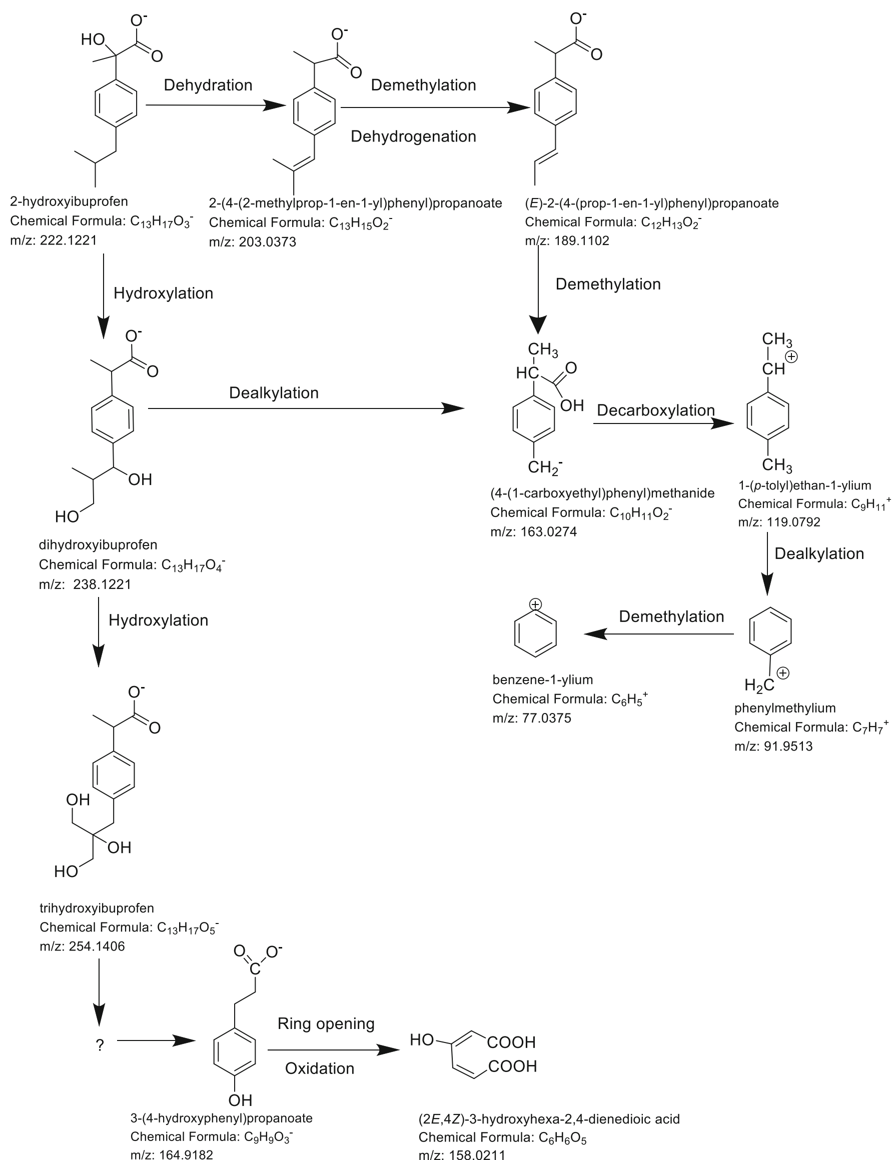

Fig. 13.6 Biodegradation pathway of pharmaceutical compound ibuprofen by *Micrococcus yunnanensis* (adapted from Sharma et al. 2019)

diclofenac was achieved by forest soil microbial consortia within 7 days (Facey et al. 2018). Ecosystem functioning may get disturbed by changing in the functional groups of microorganisms (Rodarte-Morales et al. 2012). Biodegradation is regarded as the key process for abolishing the prevalent xenobiotics including pharmaceuticals (Topp et al. 2013). Degradation of contaminants is likely to be only if the toxicity of the molecules does not obstruct the activities of microbes.

Therefore, microorganisms have a vital role in the entire processes that they dynamically maintain the functioning of ecosystem.

13.5.1 Bacteria

There are various studies that report on the degradation of environmental contaminants by bacteria. Many bacteria have been identified specifically to degrade the pharmaceuticals (Murdoch and Hay 2015; Marchlewicz et al. 2016). Degradation of pharmaceutical wastewater in a cost-effective and sustainable way can reduce their harmful impact on natural resources. Degradation process by bacteria is generally based on two mechanisms. The first one is co-metabolism in which various kinds of enzymes catalyze the metabolism of other substrate and another one is metabolic degradation in which organic pollutants are utilized as sole carbon and energy source (Tiwari et al. 2017). Zeng et al. (2009) described that heterotrophic bacteria are capable to degrade pharmaceuticals. *Pseudomonas aeruginosa* TJ1 used 17 β-estradiol (E2) as carbon and energy source for degradation. *Stenotrophomonas maltophilia* KB2 has shown the ability to metabolically degrade naproxen (Wojcieszyńska et al. 2014). Lin et al. (2015) isolated two cefalexin-degrading bacteria from activated sludge and more than 90% cefalexin degradation is obtained within a day. Co-metabolism process is done by bacteria which are generally used in wastewater treatment plant where pharmaceuticals present in even at low concentration (Onesios et al. 2009). Bacterial degradation of ritalinic acid is first time reported where different microbial strains isolated from environmental matrices (*Arthrobacter* sp., *Phycicoccus* sp. and *Nocardioides* sp.) used ritalinic acid as C and N sources (Woźniak-Karczewska et al. 2018). Some other studies related to bacterial degradation are shown in Table 13.1.

Pure culture of bacteria (*Acinetobacter* sp. and *Microbacterium* sp.) can also degrade antibiotics such as sulfamethoxazole. Some bacteria such as *Pseudomonas* sp., *Achromobacter denitrificans*, etc. need an additional carbon source to degrade pharmaceuticals (Shourian et al. 2009; Nguyen et al. 2017). The enzymatic treatment is used for bio-refractory organic compounds which are generally found in antibiotics and it works effectively even at different concentrations and broad range of pH, temperature, and salinity. *B. subtilis* 1556WTNC was proved as most potent bacteria to grow and produce the β-lactamase and degrade the cephalexin at pH range 6–7.5 at temperature (30–35 °C) (Al-Gheethi and Ismail 2014). Thus, it found suitable to treat sewage treatment producing β-lactamase that cleaves the β-lactam ring to in effective the antibiotic (Akindele et al. 2010).

13.5.2 Fungi

In wastewater treatment process for biotransformation, white rot fungi (WRF) and their oxidoreductase enzymes have been considered as cost-effective and eco-friendly solution. This group of microorganisms is capable to degrade the pollutants

Table 13.1 Degradation of pharmaceuticals by selected bacterial strains

Compound	Matrices	Country	Bacteria species	Type/mechanism of degradation	Removal of drug (%)	References
Ibuprofen	Wastewater treatment system of hospital	Netherlands	Undefined	Aerobic and anaerobic batch experiments	100	Langenhoff et al. (2013)
	Sewage sludge	USA	*Variovorax Ibu-1*	Metabolic degradation	–	Murdoch and Hay (2015)
	Soil	Poland	*Bacillus thuringiensis*	Co-metabolism	46.56	Marchlewicz et al. (2016)
Cefalexin	Sewage treatment plant	Hong Kong	*Pseudomonas* sp. *CE21*, *CE22*	Batch reactor experiments	46.7 90	Lin et al. (2015)
Naproxen			*Stenotrophomonas maltophilia KB2*	Metabolic degradation	78	Wojcieszyńska et al. (2014)
Diclofenac	Procured from market	Portugal	*Labrys portucalensis F11*	Batch reactor experiments, co-metabolism	100	Moreira et al. (2018)
Iopromide	Wastewater treatment plant	China	*Pseudomonas* sp.	Co-metabolism	88.24	Xu et al. (2014)
Sulfamethoxazole	Wastewater treatment plant	Spain	*Ammonia oxidizing bacteria*	Batch reactor experiments	86–98	Kassotaki et al. (2016)
Estrone (E1) and 17β-estradiol (E2)	Activated sludge	China	*Sphingomonas* sp.	Bacterial cell immobilization technique	87–94	Ma et al. (2016)
Enrofloxacin and ceftiofur	Procured from market	Portugal	*Achromobacter*, *Variovorax*, and *Stenotrophomonas* genera) and Bacteroidetes (e.g., *Dysgonomonas, Flavobacterium* and *Chryseobacterium* genera)	Batch reactor experiments	40–55	Alexandrino et al. (2017)

at large extent among all fungal species, such as non-steroidal anti-inflammatory drugs (Hata et al. 2010). Degradation of pharmaceuticals by fungi depends on various factors like type of target compounds, fungi species, secretion of enzymes, composition of culture media, etc. Removal of pharmaceuticals by fungi may involve two phases, first one is surface binding on fungal cell and another is metabolism dependent phase in which biotransformation takes place outside the cell by extracellular enzymatic activities (Naghdi et al. 2018). The entire fungal cell treatment can degrade the wide spectrum of drugs (anti-inflammatory, antibiotics, antiepileptics, etc.) with combined effect of intracellular and extracellular enzymes and biosorption of pharmaceuticals on the biomass (Tran et al. 2010). For example, sulfamethoxazole (SMX) is an antibiotic drug and it is a recalcitrant. Some white rot fungi *Pleurotus ostreatus*, *Pleurotus pulmonarius*, and *Trametes* sp. have shown their capability to degrade the sulfamethoxazole (de Araujo et al. 2017). Degradation of diclofenac was achieved 99% by *Bjerkandera* sp. R1 (Rodarte-Morales et al. 2012). Nowadays, enzymes excreted by white rot fungi are used for alteration of pollutants into their harmless compounds or metabolites (Arora and Sharma 2010). The example of some extracellular oxidoreductase enzymes are lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and laccase (Lac) (Garcia-Ruiz et al. 2014). Table 13.2 has depicted the capabilities of various fungal sp. to degrade pharmaceuticals.

13.5.3 Algae

Recently, algal-based technologies have gained attention due to their potency for wastewater treatment and hazardous waste removal. This is a sustainable way to remove contaminants and also sequestrate greenhouse gases (Park et al. 2011). These technologies generally remove the pharmaceuticals from wastewater in association with sorption, biodegradation, photodegradation, and volatilization (Zhang et al. 2012a, b). Algal-based technologies remove chemical oxygen demand which reduces the energy input by oxygen which is supplied through photosynthetic process (Wang et al. 2016). Current studies have reported that *Chlorella sorokiniana* have the great potential to degrade paracetamol and salicylic acid using nutrient media (Escapa et al. 2015). For the removal of micro-pollutants and pharmaceuticals, biodegradation and photolysis have been recommended as key degrading pathway (de Wilt et al. 2016). Table 13.3 shows the capability of algae to degrade the pharmaceuticals.

Algae are the point of interest as they can be used for treatment of wastewater as well as sequestration of CO_2 and production of bioenergy (Craggs et al. 2012). Tam et al. (2002) reported that biosorption is the physicochemical method which occurs on the algal cell surface which is an important removal pathway of dead and living cells. In recent times, mixotrophic microalgae have been proved a good source of biodegradation of organic and inorganic contaminants followed by utilization of biomass for sustainable bioenergy production (Pancha et al. 2015). These are fresh water microalgae which survive on nitrogen and phosphorus rich compounds and

Table 13.2 Removal of pharmaceuticals by selected white rot fungi

Compound	Matrices	Fungal species	Mechanism	Removal (%)	References
Acetaminophen	Non-sterile urban wastewater	*Trametes versicolor*	Fluidized bed reactor	100	Cruz-Morato et al. (2013)
Carbamazepine	Spiked water	*Trametes versicolor*	Fluidized bed reactor	61–94	Jelic et al. (2011)
Ciprofloxacin	Non-sterile urban wastewater	*Trametes versicolor*	Fluidized bed reactor	84.71	Cruz-Morato et al. (2013)
Diclofenac	Spiked water	*Phanerochaete chrysosporium*	Stirred tank	>99	Rodarte-Morales et al. (2012)
Ibuprofen	Spiked water	*Phanerochaete chrysosporium*	Stirred tank	75–90	Rodarte-Morales et al. (2012)
Ketoprofen	Non-sterile urban wastewater	*Trametes versicolor*	Fluidized bed reactor	100	Cruz-Morato et al. (2013)
Metronidazole	Non-sterile urban wastewater	*Trametes versicolor*	Fluidized bed reactor	85	Cruz-Morato et al. (2013)
Naproxen	Spiked water	*Phanerochaete chrysosporium*	Stirred tank	>99	Rodarte-Morales et al. (2012)
Sulfamethoxazole	Hospital wastewater	*Trametes versicolor*	Fluidized bed reactor	100	Cruz-Morato et al. (2014)
Tetracycline	Hospital wastewater	*Trametes versicolor*	Fluidized bed reactor	100	Cruz-Morato et al. (2014)

efficiently uptake the organic contaminants as a source of carbon. Some earlier studies have reported toxicological impacts of carbamazepine on microalgae and its removal (Zhang et al. 2012a, b; Matamoros et al. 2016). Xiong et al. (2017a, b) reported that *Chlamydomonas mexicana* can co-metabolize the ciprofloxacin and increase the rate of degradation from 13% to 56% within 11 days. Moreover, a study reported that 7-amino acid was completely removed by the process of hydrolysis, photolysis, and adsorption on the surface of microalgae (Guo et al. 2016). Sulfamethoxazole can be removed by algal species (e.g., *Nannochloris*) which is rarely degradable by bacteria and fungi (Bai and Acharya 2016).

Table 13.3 Degradation of pharmaceuticals by some algal species

Compound	Algal species	Drug removal (%)	Mechanism of degradation	References
17 β-estradiol	*Chlamydomonas reinhardtii*	100	Biodegradation	Hom-Diaz et al. (2015)
Estrone	*Scenedesmus dimorphus*	85	Biotransformation	Zhang et al. (2014a, b)
Progesterone	*Scenedesmus obliquus*	>95	Biodegradation	Peng et al. (2014)
Ciprofloxacin	*Chlamydomonas Mexicana*	13–56	Co-metabolite, enhanced with electron donors	Xiong et al. (2017b)
Sulfamethoxazole	*Nannochloris* sp.	32	Algal mediated photolysis	Bai and Acharya (2016)
Triclosan	*Nannochloris* sp.	100	Uptake and biodegradation	Bai and Acharya (2016)
Levofloxacin	*Chlorella vulgaris*	9.5–91.5	Biodegradation enhanced with NaCl	Xiong et al. (2017a, b)
Diclofenac	*Chlorella sorokiniana*	29.99	Biodegradation	Escapa et al. (2015)
Trimethoprim	*Nannochloris* sp.	0	Non-biodegradable	Bai and Acharya (2016)

13.6 Strategies for Future Research

Efficient treatment of pharmaceutical waste is a huge challenge because of their complex structures, hazardous characteristics. Due to their poor disposal techniques in environment drugs are released to municipal wastewater systems. Thus it creates the potential threat by addition of increased level of antibiotics in natural systems. Pharmaceutical active compounds cause their risk to the ecosystem by their constant accumulation and the probability to grow pharmaceutical resistant microbial strains. So, it is very essential to protect environmental matrices from the discharge of pharmaceutical waste. Many wastewater treatment technologies are not adequate to degrade wastewater. For effective management there are five main strategies: planning, organizing, leading, coordinating, and monitoring. However, the waste management process includes various steps such as segregation, collection of waste, transportation, storage, disposal, waste minimization, and reuse (Townend and Cheeseman 2005).

The most efficient strategy for pharmaceutical waste management is zero discharge approach. It is a green process which focuses on no generation of any waste. Although zero discharge of waste is very difficult the production of the pharmaceutical compounds by green technology which is called as green chemistry approach is

possible. By this concept, product is manufactured by harmless method and remains same till their complete life cycle (Eissen et al. 2008). By this strategy product can be made in comparatively less steps, using less energy, smaller amount of raw materials, and production of less by-products and by far they are biodegradable. Thus this approach is sustainable and environment friendly.

Additionally, green production also focuses on biotechnology for the production of biopharmaceuticals. In that process enzymes isolated from the microorganisms used as biocatalyst for the manufacturing of pharmaceutical compounds. It is more economic and eco-friendly way rather than artificial catalysts (Zaks and Dodds 1997). Due to bio-catalytic process many wastewater treatment plants now have the potential of biodegradation. With the use of membranes waste can be collected at large scale which eases the solid waste disposal.

For the management of waste industries, they should be also hold up for setting up of waste management teams, drafts documentation and waste management plan implication. There should be some incentives, formation of industrial disposal units, and free training for the staff to promote waste management. Government should also take action to make and imply the policies regarding pharmaceutical waste management.

13.7 Conclusion

A wide variety of pharmaceuticals are present in the environmental matrices due to their inadequate discharge to the environment or inefficient removal process. Even low concentrations of pharmaceuticals can cause harmful impact to the ecosystem, so proper elimination and management of these compounds are very important. Biodegradation is one such sustainable technique to degrade the pharmaceuticals. The proportion at which pharmaceuticals are eliminated from the environment depends on the occurrence of indigenous microbial communities which are able to degrade them. Most of the pharmaceutical groups are easily bio-transformed by metabolic or co-metabolic pathways. Environmental factors extensively affect the rate of transformation and bioavailability of microbial degradation. For the management of pharmaceutical waste some practical approaches should be employed at very urgent basis. Government should also effectively establish the inexpensive and eco-friendly collection and disposal systems of pharmaceuticals. Appropriate disposal techniques for drugs can facilitate the reduction in environmental burden of pharmaceuticals. To decrease the load of pharmaceuticals in environment all stakeholders, NGOs, government, researchers, pharmacist and public should come jointly and take the action. Thus, adequate environmental strategies are required to eliminate the exposure of pharmaceuticals for environmental and health safety.

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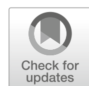

Metabolism of Dioxins and Dioxins-Like Compound, Its Regulation and Toxicological Pathways

14

Prashant Kumar Jaiswal and Jyotsana Gupta

Abstract

Dioxin and dioxin-like compounds are group of chemical compounds that are highly toxic, persistent environmental pollutants that can affect human health, wildlife and environment adversely. Dioxins are ubiquitous in environment (soil, water and air) and are formed mostly as by-products of various industrial activities such as metallurgy, waste incineration or burning. Environmental clean-up of dioxins is extremely difficult partly because of its planner structure, extremely hydrophobic nature, strong adsorption/absorption to the soil. Once released into the environment dioxins can contaminate the human and animal food supply. Dioxin mediated toxicity can result in immune-toxicity, induction of enzyme related disorders, chloracne, carcinogen and birth defects. This chapter details the structure, source, fate, impact on human health and environment, metabolism, biodegradation, enzymes and the genes involved during the biodegradation of dioxin and dioxin-like compounds such as dibenzofuran.

14.1 Introduction

Dioxins and dioxin-like compounds are highly toxic and hazardous persistent organic pollutants (POPs) that are known to adversely affect not only the human health but also the environment around the universe. Dioxins are biaryl ethers that can be transported by environment such as air, water, soil and once generated in one part of the globe can adversely affect the people and wildlife even far from the source of origin (EPA 2019). Dioxins are formed mostly as by-products of various anthropogenic activities including incineration, industrial processes, high-temperature processes and production of pesticides. Some of the examples include combustion of waste generated from hospital, municipal and hazardous activities as well as

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emission from automobile emissions and burning of wood, tar and coal, etc. (Alcock and Jones 1996; Kuehl et al. 1987; Thacker et al. 2007).

As a potent POP and toxicant, it has been a growing concern due to its resistance to any mode of environmental degradation processes. Dioxins have the ability to bioaccumulate due to their persistence property that in turn lead to hazardous effects on health of human/animal and the environment. In general, Stockholm Convention (2001) has explored and resolved the elimination or severely restriction on the production on POPs that are hazardous to human as well as environment (Stockholm Convention 2001).

Dioxins mediated toxicity have been well explored by various workers indicating its association with several adverse effects in human health, including but not limited to immune-toxicity, induction of enzyme related disorders, chloracne, carcinogen that can induce tumour promotion and progression and increase in developmental birth defects that can lead to stillbirths. Also, the animal derived food is one of the major sources of dioxins exposure (White and Birnbaum 2009).

14.2 Dioxins and Dioxins-Like Compound

'Dioxins' also referred as POPs, share backbone of chemical structures which is essential of its toxicity and persistence. There are several hundred types of these compounds have been found but broadly, it can be categorized into three categories: Halogenated dibenzo-p-dioxins (DD), halogenated dibenzofurans (DF) and polychlorinated biphenyls (PCBs).

Among all dioxins, 2,3,7,8-tetrachlorodibenzo-p-dioxins (TCDD) is the most potent compound (congener) of dioxins as shown in Fig. 14.1. In at least two incidences, it brought public attention. Firstly, it was used as herbicide in the name of Agent Orange in US military operation 'Ranch Hand' during the Vietnam War from 1961 to 1971 (Ngo et al. 2006). Secondly, during Seveso disaster in Italy it was released into the environment on July 10, 1976 (Eskenazi et al. 2004). Also, it is

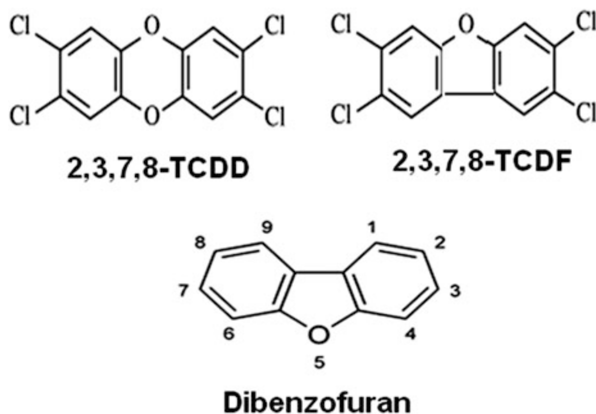

Fig. 14.1 Chemical structure of 2,3,7,8 TCDD, 2,3,7,8 TCDF and Dibenzofuran

a carcinogenic environmental contaminant that is usually present in a complex mixture of dioxin-like compounds and is responsible for various adverse effects.

14.3 Sources

Because of awareness and regulatory bodies, many countries have restricted their production of manufactured products with PCBs (EPA 2019). Halogenated especially brominated or chlorinated DDs and DFs are not formed intentionally; however, these are formed due to various anthropogenic activities, i.e. burning of trash. In addition to this, natural processes such as forest fires can also trigger formation of dioxins. Broadly, sources can be identified as following:

14.3.1 Industrial Activities

Dioxin is a contaminant formed as a by-product during production/synthesis of few chlorinated organic compounds, such as silvex (a commonly used herbicides). In recent years, regulatory agencies have been putting lots of efforts to reduce/eliminate the formation of dioxin. Although the concentrations of dioxins have reduced significantly in last three decades, however, a larger portion of the dioxins exposure in the environment is mainly due to previous releases that has occurred years ago (EPA 2019).

14.3.2 Burning

Municipal combustion processes (which use waste incineration and/or from burning fuels such as oil, coal or wood) are another source of dioxins formation. EPA (2006) summarizes that anthropogenic emissions are the major source for existing exposure in the USA, but also without ruling out the possibility of natural sources (EPA 2019). Burning of household waste or backyard burning can also lead to the formation of dioxins.

14.3.3 Industrial Bleaching

As per practice, chlorine bleaching based on various industrial processes is also responsible for production of dioxins leading to environmental hazard.

14.3.4 Smoking

Cigarette smoke is also responsible for production of dioxins in minute quantities.

14.3.5 Drinking Water

Dioxin can accumulate in potable water from incineration. Dioxins accumulated in potable water get deposited into water bodies. Also effluents and chemical industry waste containing dioxins might pollute the potable water.

14.4 Fate of Dioxins in Environment

Dioxins and dioxins-like compounds exhibit high lipid solubility which leads to the bioaccumulation in fatty tissues. Also, these halogenated dioxins exhibit enhanced stability, resistance of C–Cl bond hydrolysis and photolytic degradation. As, the stability and lipophilicity correlate with degree of halogenation, Dioxins are a topic of concern because dioxins exert adverse impact on environment by long range transport, that allow dioxins to travel and spread far from the source, and its bioaccumulation, that concentrates to potentially dangerous levels (Walker 2001).

14.5 Impact of Dioxin on Human Health

There have been a series of incidents which brought public attention where human being has been exposed to dioxins and dioxin-like compounds. In 1976, Seveso (Italy) accidental release of dioxins led to various symptoms such as multiple myeloma and myeloid leukaemia, developmental effects, chloracne (Mocarelli et al. 2008). Also, Triangle of death (a polluted land in Italy) has accumulated years of waste disposal due to organized crime and illegal activities now contaminated with TCDD (highly toxic dioxin). In 2004, Viktor Yushchenko (presidential candidate) of Ukraine was poisoned with TCDD (blood TCDD level—108,000 pg/g fat) and he suffered from chloracne with several medical complications (Sorg et al. 2009).

14.6 Metabolism of Dioxins in Mammals

TCDD and dioxin-like compounds interact with aryl hydrocarbon (Ah) receptor and it can modulate several hundred of genes related to enzymes that can activate the hydrolysis/breakdown of toxic compounds, e.g. carcinogenic benzo(a)pyrene. The Ah receptor (transcription factor) present in cell is a phylogenetically highly conserved in vertebrates, and the ancient analogs of Ah receptor are important regulatory proteins that are present in primitive species (Wittsiepe et al. 2007). The genotoxic and mutagenic effects of TCDD are still being a topic of controversy, however, involvement of TCDD/F in development of cancer has already been confirmed probably by inducing oxidative stress and the DNA damage.

14.7 Biodegradation of Dioxins and Dioxins-Like Compound

Previous literature suggests that during chlorine cycle, TCDD/F compounds undergo biodegradation in environment. The aerobic bacteria from the genus *Burkholderia*, *Pseudomonas* and *Sphingomonas* can degrade the lower chlorinated dioxins. Various studies have shed light on the co-metabolism of monochlorinated dioxins and unsubstituted dioxin. This co-metabolism has been reported to be the primary substrate. It has been documented that the degradation is initiated when the ring adjacent to the ether oxygen is attacked by a unique angular dioxygenases. Furthermore, chlorinated dioxins can also be degraded co-metabolically by white-rot fungi under aerobic conditions since it utilize extracellular lignin degrading peroxidases.

Degradation of dioxins is difficult partly because of its hydrophobic nature, planner structure, strong absorption and adsorption to soil and sediment, which results into limited bioavailability and pronounced resistance to attack by aerobic and anaerobic microorganisms (Field and Sierra-Alvarez 2008). The resistance to degradation by microorganisms could be due to lack of selective pressure related to the concentration and availability of compound. Therefore, there has been a need to find indigenous soil microflora exposed to the pollutant concentrations, which is low in agricultural applications and high at industrial effluent and municipal sludge sites (Thomas et al. 1996).

From the last decade, DF a relatively soft carbon source than chlorinated dioxins has been used as a model compound to study the degradation of dioxin. DF (just like dioxins) is a less bioavailable, resistant and hydrophobic molecule that has a planner structure.

DF is a 2, 2-biphenylene oxide having two benzene rings joined with one ether linkage. Some studies have reported that few strains of the bacteria such as *Pseudomonas,* i.e. HH69, CA10; *Brevibacterium*, *Terrabacter*, *Sphingomonas, Xanthomonas maltophilia* and *Serratia marcescens* ISTDF2 1 can lead to DF degradation (Fortnagel et al. 1990; Strubel et al. 1991; Omori et al. 1997; Harms et al. 1995; Wittich et al. 1992; Ishiguro et al. 2000; Sato et al. 1997; Jaiswal and Thakur 2007). These studies have revealed that oxygenation at ether linkage (angular oxygenation) is common among all above-mentioned bacterial strains as initial biotransformation step. 2, 2′, 3-trihydroxybiphenyl are formed via angular dioxygenation of DF just after unstable phenolic hemiacetal as first stable intermediate metabolite (Fortnagel et al. 1990; Bunz and Cook 1993; Harms et al. 1995). The cleavage in 2, 2′, 3-trihydroxybiphenyl leads to formation of 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2, 4-hexadienoic acid which is hydrolysed to form salicylic acid and 2-hydroxypenta-2,4-dienoic acid. Hydrolysis of salicylic acid forms catechol and finally, cis, cis-muconate or 2-hydroxy muconic semialdehyde are formed after dihydroxylation which is absorbed in tricarboxylic cycle (Fortnagel et al. 1990).

FA-HZ1 (*Pseudomonas aeruginosa*) isolated from landfill leachate is known to be a DF degrading bacterium. Complete genome sequence of FA-HZ1 demonstrated that it has only one circular chromosome and the complete genome has been sequenced to investigate the molecular mechanism of DF degradation (Ali et al.

2017). A recent study has characterized the strain FA-HZ1 from waste water. It has been demonstrated that during DF degradation several biodegradation intermediates are formed and the conversion of DF to 1,2-dihydroxy-1,2-dihydrodibenzofuran requires a novel enzyme called HZ6359 dioxygenase (Fawad et al. 2019).

14.8 Biodegradation of Dibenzofuran

Cunninghamella elegans and *Beijerinckia* (B8/36, a mutant strain) can oxidize DF to 2,3-dihydroxy-2,3-dihydrodibenzofuran (Cerniglia et al. 1979). The bacterial metabolite thus formed was extremely unstable. It can be converted into a mixture of 2- and 3-hydroxydibenzofuran in the presence of acid rapidly. Contrary to this, *C. elegans* formed a stable 2,3-dihydroxy-2,3-dihydrodibenzofuran intermediate. This intermediate can yield 2- and 3-hydroxydibenzofuran only under acidic conditions. The contrary results are due to formation of cis- and trans-isomers of 2,3-dihydroxy-2,3-dihydrodibenzofuran by *Beijerinckia B8/36* and *C. elegans,* respectively. Also, *C. elegans* oxidizes dibenzofuran to 2-hydroxydibenzofuran and 3-hydroxydibenzofuran. During these conditions dehydration of trans-dihydrodiol is not promoted thus implicating the formation of dibenzofuran- 2,3-epoxide after oxidation of dibenzofuran by fungus. Cis-1,2-dihydroxy-1,2-dihydrodibenzofuran is an unstable dihydrodiol produced by *Beijerinckia B8/36*. Therefore, it is evident that fungus and bacteria carry out dibenzofuran oxidation by different mechanisms. There are some studies available about *Phlebia lindtneri* (white-rot fungus) in which oxidation of DD and DF to 2-hydroxy-DD, and 3-hydroxy-DF catalysed by cytochrome P-450 monooxygenase introduces hydroxyl group to its substrates; however, the alkalophilic environment and extreme condition in contaminated site would not allow the fungus survival (Mori and Kondo 2002). Bacteria are known for its metabolic diversity and can survive up to extreme condition as well. So bacteria are better promising options for degradation of DF including dioxins.

In 1989, the microorganisms that can metabolize DF were described by Strubel et al. and Fortnagel et al. DPO1361 from *Terrabacter* sp., HH69 from *Sphingomonas* sp. and DBF 63 from *Staphylococcus auriculans* (Schmid et al. 1997; Strubel et al. 1989; Harms et al. 1995; Fortnagel et al. 1990; Kasuga et al. 2001; Schmid et al. 1997) can convert DF to two key intermediates (2,2′0.3-trihydroxybiphenyl and salicylic acid) through a novel angular dioxygenation mechanism. Contrary to the above-mentioned strains, *Sphingomonas* sp. RW1 (river Elbe) can degrade DF and also mineralize DD (Wittich et al. 1992; Harms et al. 1990; Chai et al. 2016). It has been reported that the compound DD can only be co-metabolized using other strains.

The first step in DF metabolism is formation of phenolic hemiacetals where stereospecific angular dioxygenation of the aromatic rings is responsible further on rearomatize to yield trihydroxylated intermediates (2,2′,3-trihydroxybiphenyl and 2,2′,3-trihydroxydiphenyl ether). These intermediates undergo extradiol ring cleavage same as bacterial metabolism of biphenyl (ring cleavage of 2,3-dihydroxybiphenyl) (Eltis et al. 1993). 2,2′,3-trihydroxybiphenyl undergo the ring

cleavage to produce 2-OH-HOPDA [2- hydroxy-6-(2-hydroxyphenyl)-6-oxo-hexa-2,4-dienoic acid] (Strubel et al. 1991). The 2-OH-HOPDA is hydrolysed by a hydrolase (2-hydroxy-6-oxo-6-phenylexa-2,4-dienate) to form salicylate and 2-hydroxypenta-2,4-dienoate (Bunz and Cook 1993). This hydrolysis is same as seen during bacterial metabolism of biphenyl (hydrolysis of 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoate). However, the ring-cleavage product 6-(2-hydroxyphenyl)ester of 2-hydroxymuconic acid formed from 2,2′,3-trihydroxydiphenyl ether undergoes hydrolysis to form 2-hydroxymuconate and catechol (Wittich et al. 1992).

Thus, hydrolysis of DF and dioxin by enzymes form salicylate and 2-hydroxypenta-2,4-dienoate or catechol and 2-hydroxymuconate. During degradation, either *meta*-cleavage pathway or *ortho*-cleavage pathway of catechol is induced. Meta-cleavage pathway of catechol induced during degradation of DF and dibenzo-p-dioxin DD by RW1 forms two intermediates 2-hydroxymuconate and 2- hydroxypenta-2, 4-dienoate (Whitman et al. 1992; Lian and Chapman 1993). On the other hand, *ortho*-cleavage of catechol is induced during DD degradation. Thus, the degradation of catechol can be accomplished by either *meta*-cleavage or *ortho*-cleavage; however, there is paucity of information about relative importance of these routes. During DF degradation, catechol *meta*-cleavage and gentisate pathways are induced. However, degradation of salicylate via catechol (salicylate 1-hydrolase) or via gentisate (salicylate 5-hydrolase) pathway has not been clearly understood.

Strain XLDN2–5 (*Sphingomonas* sp.), a carbazole utilizing bacteria isolated from petroleum-contaminated soil utilizes carbazole as a sole source of carbon, nitrogen, and energy and can degrade DF and dibenzothiophene. XLDN2–5 utilizes angular dioxygenation pathway to degrade DF to salicylic acid via an intermediate 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienic acid. Also, strain XLDN2–5 degrades DT through ring cleavage and sulphoxidation pathways. This strain can co-metabolically degrade both DF and DT using carbazole as a substrate. When strain XLDN2–5 was incubated for 40 hr. with DT and DF; DT, DF and CA were degraded about 90%, 100% and 100%, respectively suggesting its important role in the bioremediation of environment/surrounding contaminated by these compounds (Gai et al. 2007).

Furthermore, strain JB (*Comamonas* sp.) can co-metabolically degrade DF and dibenzothiophene. Naphthalene is the primary substrate. It was observed that DF was degraded to 1,2-dihydroxydibenzofuran then to 2-hydroxy-4-(3′-oxo-3’H-brnzofuran-2′-yliden)but-2-enoic acid that was finally degraded to catechol. During the degradation several naphthalene degrading enzymes were detected (Ji et al. 2017).

Strain RW1 (*Sphingomonas* sp.) and strain JWS (*Burkholderia* sp.) consortium can mineralize the 4-chlorodibenzofuran via distal dioxygenation by attacking the unsubstituted aromatic ring adjacent to ether bridge to yield 3(prm1)-chloro-2,2 (prm1),3-trihydroxybiphenyl. Further, this compound undergoes meta-cleavage to form an intermediate that is hydrolysed to form a C-5 moiety. Later on this C-5

moiety is degraded to form a Kreb's cycle intermediate and 3-chlorosalicylate that are released into the culture medium. Thus a co-culture of strain RW1 and strain JWS is capable of completely degrading 4-chlorodibenzofuran (Schindowski et al. 1991; Arfmann et al. 1997).

In angular pathway, metabolites f, n, p, z3, z4, z5 and z6 are formed, whereas metabolites z, z1 and z2 are formed non-enzymatically reported in *Sphingomonas* RW1, *Terrabacter* DBF63 and *Pseudomonas* sp. CA10. Lateral pathways run through L, x, z4 and t; or m, u, w, z4 intermediary metabolites in *Ralstonia* sp. Strain SBUG 290. Metabolites B, c, d, g, h, I, j, k, q, r, s and y are found (Gesell et al. 2004). In DF degradation, the angular pathways are more common. However, very few reports are available which suggested that some bacteria are able to degrade by using lateral pathway such as Strain SBUG 290 from *Ralstonia* sp. (Becher et al. 2000). Not only this, some reports have shown mono-hydroxylation as well as formation of conjugates with ribose sugar in DF. All reported pathways have been put together in Fig. 14.2.

Nojiri and Omori (2002) have reviewed thoroughly the molecular basis of bacterial degradation of dioxins under aerobic conditions and investigated the role of angular dioxygenation. In this study it was mentioned that dioxin-degrading strains can actually function in dioxin-contaminated soil, thus providing a bioremediation method for dioxin contamination. As mentioned earlier some studies are also available about white-rot fungus *Phlebia lindtneri* that can oxidize DD and DF to 2-hydroxy-DD, and 3-hydroxy-DF by a cytochrome P-450 monooxygenase (Mori and Kondo 2002).

14.8.1 Enzymes Involved in Degradation of Dibenzofuran

Aromatic compounds are degraded by initiation of ring-activating reaction. During dioxygenolytic activation, two hydroxyl groups are incorporated at the *ortho* positions of the aromatic ring. The *cis*-dihydrodiol dehydrogenases rearomatize *cis*-dihydrodiol formed via dioxygenolytic activation. However, in case the aromatic ring is substituted, the dioxygenation reaction can incorporate the hydroxyl group in two different ways either at the substituted carbon atom and its neighbouring carbon atom or on two adjacent unsubstituted carbon atoms. In case of incorporation at substituted carbon atom and its neighbouring carbon atom, the reaction can be concomitant with the spontaneous elimination of the substituent.

2-chlorobenzoate; 1,2,4,5-tetrachlorobenzene; 2,2′-dichlorobiphenyl or sulphoaromatics (haloaromatics) form an unstable dihydrodiol analogues during degradation process (Engesser et al. 1989; Beil et al. 1998; Haddock et al. 1995). A dehydrogenase is not required during degradation as it undergoes spontaneous rearomatization. Similarly, angular dioxygenation of biarylethers forms hemiacetals (an unstable dihydrodiol analogues) during degradation which undergo spontaneous rearomatization to form two monocyclic aromatics from simple biarylethers or dihydroxybiphenyl derivatives from DF and DD, respectively (Engesser et al. 1990; Schmidt et al. 1992). Catalysis by ring hydroxylating dioxygenases requires

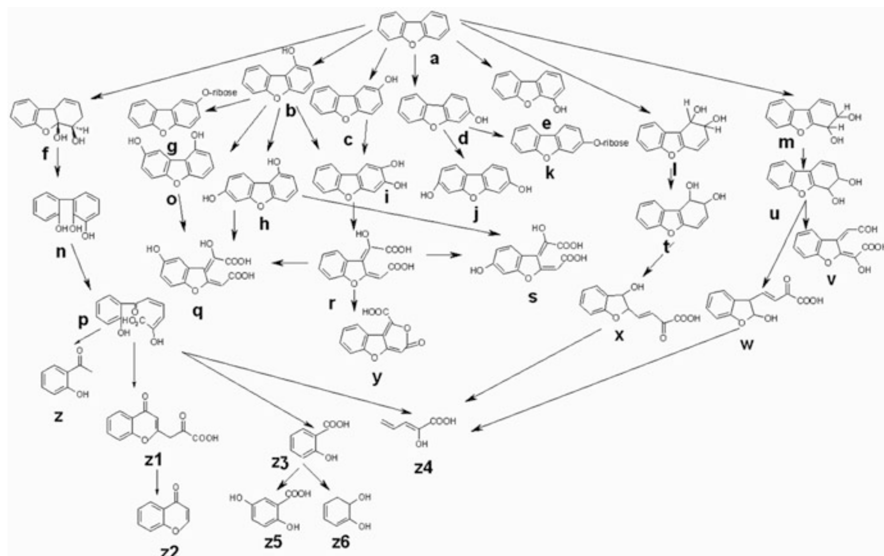

Fig. 14.2 Multiple pathways for dibenzofuran degradation: Dibenzofuran {product A} Mono hydroxy dibenzofuran {product b, c, d and e}; 2,2′,3-trihydroxybiphenyl {n}; 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4–5 hexadienoic acid (2′-OH-HOPDA) {product P}; 2′-Hydroxy aceto phenone{product z}; 3-(Chroman-4-on-2-yl)pyruvic acid {product z1}; chromone {product z2}; Salicylic acid {product z3}; 2,4-hexadienoic acid {product z4}; Gentesic acid {product z5}; Catechol {product z6}; 1,2-dihydro-1,2-dihydroxydibenzofuran {product u}; 3,4-dihydro-1,2-dihydroxy dibenzofuran {product l}; 2'hydroxy-4-(3′-oxo-3'H-benzofuran-2′-yliden)but-2-enoic acid or (3′-HOBB) {product x}; 2′hydroxy-4-(2′-oxo-2'H-benzofuran-3′-yliden)but-2-enoic acid or (2′-HOBB) {product w} and 2-(1-carbonyl methylidine)-2,3-dihydrobenzofuran lidene-glycolic acid {product v}. 1-hydroxydibenzofuran {product b}; 2-hydroxydibenzofuran {product c}; 3-hydroxydibenzofuran {product d}; 4-hydroxydibenzofuran {product e}; riboside conjugate of 2-hydroxydibenzofuran {product h}; riboside conjugate of 3-hydroxydibenzofuran {product k}; 2,8-dihydroxydibenzofuran {product o}; 2,7-dihydroxydibenzofuran, {product g}; 3,7-dihydroxydibenzofuran {product I}; [2-(1-carboxymethylidene)-6-hydroxy-benzofuran-3-ylidene]-hydroxyacetic acid {product S};benzo[b]furo[3,2-d]-2-pyrone-6-carboxylic acid {product y}

oxygen, Fe^{2+} and reduced pyridine nucleotides. Ring hydroxylating dioxygenases are soluble, multicomponent enzyme systems that are comprised of two or three separate proteins. These dioxygenases consist of an electron transport chain where electrons from NAD(P)H are channelled to the catalytic terminal oxygenase component for substrate transformation. The catalytic terminal oxygenase component contains a cluster [Rieske (2Fe-2S)] and mononuclear iron called Rieske non-haeme iron oxygenase (Gibson and Parales 2000).

Generally, the electron transport chain consists of either an iron–sulphur flavoprotein reductase or a flavoprotein reductase and an iron–sulphur ferredoxin; however, the structure of ring hydroxylating oxygenase is considerably different. These enzymes have been classified as Class I, II, III based on the variation in number and the size of constituent compounds and their nature of redox centre (Batie and Kamin

1986; Batie et al. 1992). The Class I and III dioxygenases, reductases contain a flavin chromophore (FAD—Class IA and Class III or FMN—Class IB as a prosthetic group) and a plant-type iron–sulphur [2Fe-2S] cluster. An additional ferredoxin component carrier along with a Rieske-type iron–sulphur cluster [2Fe-2S] is present in Class III that functions as an intermediate electron-transfer. Class II enzyme reductases contain FAD and either a plant-type or Rieske-type (Class IIA, Class IIB, respectively) [2Fe-2S] iron–sulphur cluster. Limitation of this classification is that it only considers electron transport components and eliminates other important information that should be taken into account such as information on oxygenase. For example, a monooxygenase reaction catalysing enzyme (2-oxo-1,2-dihydroquinoline monooxygenase) and a dioxygenase reaction catalysing enzyme (benzoate dioxygenase) are classified as Class IB, due to presence of iron-flavoprotein reductase (Rosche et al. 1995). On the other hand, these monooxygenase and dioxygenase differ in composition, i.e. $(\alpha)_6$ and $(\alpha\beta)_3$ subunit, respectively. Since the catalytic α-subunit of oxygenases defines the substrate specificity it should be considered as superfamily (Beil et al. 1998; Mondello et al. 1997; Gibson and Parales 2000).

Dibenzofuran 4,4a-dioxygenase is responsible for the stereospecific angular dioxygenation of DF and DD in RW1 (*Sphingomonas* sp.). The dibenzofuran 4,4a-dioxygenase was supposed to be a heterodimer ($\alpha_2\beta_2$) with molecular weights of α subunit and β subunit, 45 and 23 kDa, respectively. It has been confirmed by spectroscopy that the α subunit of a Rieske-type (2Fe-2S) cluster is contained in oxygenase subunits of ring-activating oxygenases. However, the N-terminal sequence of α subunit of dioxin dioxygenase exhibits significant differences when compared to other dioxygenases. In in vitro studies it is clear that DF (into 2,2′,3-trihydroxybiphenyl) and DD (into 2,2′,3-trihydroxybiphenyl ether) are degraded by dibenzofuran 4,4a-dioxygenase that consists of an oxygenase and electron transport chain. The process of degradation introduces two oxygen atoms from a O_2 molecule that are added to two vicinal carbon atoms of which one is involved in the bridges between the two aromatic rings. The purified RedA1 and RedA2 are isofunctional flavoproteins that transfer electrons from NADH to ferredoxin and oxygenase component (Bunz and Cook 1993).

The carbazole (1,9a-dioxygenase) characterized from strain CA10 (*Pseudomonas* sp.) is capable of catalysing an angular dioxygenation similar to dibenzofuran 4,4adioxygenase of RW1 (Nojiri and Omori 2002).

14.8.2 Genes Involved in Degradation of Dibenzofuran

The catabolic genes involved in the metabolism of DF have been already reported in many strains such as *Sphingomonas* HH69, *Sphingomonas* sp. RW1, *Pseudomonas* sp. strain CA10, *Terrabacter* sp., *Pseudomonas aeruginosa* and *Xanthomonas maltophilia* (Harms et al. 1995; Wittich et al. 1992; Sato et al. 1997; Omori et al. 1997; Schmid et al. 1997; Ishiguro et al. 2000; Coronado et al. 2015) and many more. However, catabolic genes of very few strains have been well explored.

Sequence analysis has confirmed that the genes coding for the enzymes involved in DF degradation are dispersed vastly on chromosome. Armengaud et al. (1999) have presented the genetic organization of the enzymes with several loci. The α and β subunits of dibenzofuran 4,4a-dioxygenase, DbfB and H1 hydrolase are mapped to *dxnA1A2*, *dbfB* and *dxnB* gene from Locus A. Locus B and C are associated with *fdx1* and *redA2* genes encoding, respectively the ferredoxin and reductase of the RW1 dibenzofuran dioxygenase. Cosmidic library of RW1 has been hybridized with a probe (obtained using two primers) to identify the Locus D. These primers have been designed based on DxnA1 N-terminal end of conserved motives in Class II B dioxygenases. These primers were designed to amplify *dxnA1A2*; however, they amplified a short fragment carrying an ORF that encode a polypeptide. Thus demonstrating the identity to another ring-hydroxylating dioxygenase (α-subunit). Later on, analysis of *edo2* gene sequence harboured on Locus E, identified additional ORFs suggesting ring-hydroxylating dioxygenases (α and β subunits) share homology with ORFs G1, G5, G6. It was found that the Locus E harbour several degradative genes that also include monooxygenase (ORF G4). The ORF G4 polypeptide sequence was found to have similarity with several flavin-containing monooxygenases when isolated from different sources. The ORF G2 (*edo2*) and ORF G4 have similarities to Rieske-type (2Fe-2S) ferredoxins (Class IIB and III ring-hydroxylating dioxygenases). G4 is located to downstream of ORF encoding G7 and it shows homology to HOPDA hydrolases.

This demonstrates that the genes *dxnA1A2* and *dbfB*, *dxnB* and *dxnC* are located on Locus A. *dxnB* is located downstream of *dxnC*(first ORFs) in a compact operon (Armengaud et al. 1999). Of the ten ORFs first three are *dxnC*, *ORF2* and *fdx3*. The ORF of *dxnC* encodes a protein that has very low homology to bacterial siderophore receptors, *ORF2* encodes a polypeptide that represents insignificant homology to known proteins and *fdx3* encodes a putative ferredoxin. In vitro assays have demonstrated that RedA2 reduces the Fdx3. This has been further confirmed by in vivo assays where co-expression of both *fdx3* and *redA2* with *dxnA1A2* has confirmed that Fdx3 can serve as an electron donor for the dioxin dioxygenase. This ferredoxin and Fdx1 have similar midpoint redox potential. The gene *dxnD* downstream of *fdx3* gene encodes a phenol monooxygenase-like protein (69- kDa) that has an activity for the turnover of 4-hydroxysalicylate. The gene *dxnD* is followed by *dxnE* that encodes a 37-kDa protein. This protein shares a similarity in sequence and activity to maleylacetate reductases. Following *dxnE* is *dxnF* that encodes an intradiol dioxygenase (33-kDa) that cleaves hydroxyquinol to produce maleylacetate.

Further to *dxnF* is *dxnGH* that encode a heteromeric protein is a 3-oxoadipate succinyl-CoA (coenzyme A) transferase. Following *dxnGH* is *dxnI* that encode a protein that exhibits remarkable homology to thiolases (acetyl-CoA acetyltransferases) (Armengaud et al. 1999). Finally the last ORF encodes a putative transposase. Combining together, the five ORFs- *dxnD*, *dxnF*, *dxnE*, *dxnGH* and *dxnI* form a 4-hydroxysalicylate/hydroxyquinol degradative pathway.

Thus *Sphingomonas* sp. RW1 is genetically diverse with four different α-subunits of ring-hydroxylating dioxygenase, two α-subunits, two reductases, three

ferredoxins, three *meta*-cleavage enzymes. The genes encoding these enzymes are dispersed among several gene clusters. Similar findings have been observed in case of *Sphingomonas aromaticivorans* F199 as well (Romine et al. 1999) that contain several oxygenase subunits an important characteristic of *Sphingomonas* sp. Furthermore, the gene products can combine in a random fashion to form hybrid aromatic oxygenase. So far, there is lack of information on several groups of isoenzymes of strain RW1 (*Sphingomonas* sp.). They might play an important role in different metabolic pathways.

The RW1 (*Sphingomonas* sp.) can use dioxin and DF as a sole carbon source; however, it cannot grow on chlorinated dioxins and DF. It could be due to limited substrate range of initial dioxin dioxygenase, formation of dead end products and toxic intermediates. 3-chlorocatechol, the intermediate formed has been found to inhibit extradiol ring-cleavage dioxygenases (Bartels et al. 1984). Catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 is the best example where the mechanism of inhibition has been studied (Bartels et al. 1984).

Genetic structure of catabolic genes involved in DF degradation of CA10 (*P. resonvorans*) has been also well characterized. Upstream flanking region of carBC gene of CA10 has two ORFs (ORF4 and ORF5). Similarly, downstream region of carBC gene has three ORFs (ORF6, ORF7 and ORF8). When sequenced, ORF6 demonstrated homology with ferredoxin and ORF8 showed homology with ferredoxin reductase component of bacterial multicomponent dioxygenase system. ORF4 and ORF5 share sequence homology and the amino acid sequence demonstrated approximately 30% homology with alpha subunit (large subunit) of terminal oxygenase component.

ORF4 and ORF-5 (product-terminal dioxygenase), ORF6 (product-ferredoxin) and ORF8 (product-ferredoxin reductase) of CARDO (carbazole 1,9a-dioxygenase) attack the angular position adjacent to the nitrogen atom of carbazole. However, the product of ORF7 is not indispensable for CARDO activity. Hence, ORF4, ORF5, ORF6 and ORF8 were designated as carAa, carAb, carAc and carAd, respectively.

The products of carAa (ORF4), ORF7 and carAd (ORF8) have a molecular weight of 43, 36 and 11 kDa, respectively as revealed by SDS-PAGE. However, the product of carAc was not visible on SDS-PAGE. CARDO can oxidize several polyaromatic compounds that include DD, DF, biphenyl and polycyclic aromatic hydrocarbons (naphthalene and phenanthrene). DD and DF form 2,2′,3-trihydroxydiphenyl ether and 2,2′,3-trihydroxybiphenyl metabolites and thus it has been concluded that CARDO attacks at the angular position is adjacent to the oxygen atom of DD and DF similar to carbazole (Nojiri and Omori 2002).

The genes dbfA1A2 (oxygenase component of multicomponent dioxygenase), dbfBC (meta-cleavage enzyme and hydrolase) and pht (phthalate-degrading enzymes) of strain DBF63 (*Terrabacter* sp.) clearly reveal an important role in aromatic compound degradation. The DBF63 cells when grown on DF and/or fluorene clearly demonstrated that the DbfA1A2 and DbfBC are responsible for catalysing the conversion of DF to salicylate and both DbfA1A2 and Pht enzymes are involved in degradation of fluorene. In vitro experiments have confirmed that dbfA1A2 cistron and pht operon are located on pDBF1–160 kb and pDBF2–190 kb

(two linear plasmids), whereas dbfBC genes are located on chromosome. Since pht operon is located upstream to dbfA1A2 cistron, the dioxin-catabolic genes were dispersed on the genome. Contrary to this, FN-catabolic genes were gathered on plasmids.

14.9 Conclusion

Growing evidence suggests that even at extremely lower dose, dioxins and dioxins-like compounds can be a potent carcinogen that interferes in endocrine system which leads to major cause of concern. Its universal presence, exhibiting high lipid solubility and lower water solubility causes bioaccumulation in fatty tissues and its persistence in the environment results in slower elimination in animals and environment. Environmental clean-up of dioxins is extremely difficult partly because of its planner structure, extremely hydrophobic nature, strong adsorption/absorption to the soil resulting in limited bioavailability and pronounced recalcitrance to attack by microorganisms (aerobic and anaerobic). Therefore, in conlusion, the enrichment of bacteria metabolizing - Dibenzofuran as a model compound with similar structure and phyisico-chemical properties, could be very useful for systematic environmental clean-up of dioxins.

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Film Based Packaging for Food Safety and Preservation: Issues and Perspectives

15

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Abstract

The extensive production of conventional plastics and their use in various food industries as packaging materials create a significant threat to the environment. This ends up creating problems concerned with performance, processing, and overall cost, thus being a big question in dealing with these non-renewable materials. The bioplastics evolved during development of renewable resources. As a part of the consequences to the dynamic changes in the present demand of customer and market scenario, the film-based active packaging system is of huge importance. The application of packaging systems is not to pose as a "wrap on," hence lowering the quality control. It should, anyway, serve as an "add on" for the protective measures taken to assure the safety and best quality of foods. This chapter aims to compile information on types of active food packaging systems, its commercial applications meant for improving food safety and quality with the extension of its life. It also describes various critical factors to be considered for commercialization, current market strategy, and legislative considerations, and application of bioplastic as packaging materials to meet ever-growing consumer demands with comparatively high quality fresh produce.

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15.1 Introduction

Microbial growth on food products leads to spoilage of many foods. During storage, the growth of undesirable microorganisms should be controlled either by coating antimicrobial substances on the food surface or incorporating these substances into food packaging materials. Employment of active packaging happens to be a new approach in food preservation, thereby enhancing the safety margin, reassuring the high quality of products, and incorporating antimicrobial agents in films based on polymers can be used as active packaging (Reyhan and Ozlem 2015; Ishrat et al. 2018). Antimicrobial packaging stands as one of the unconventional packaging concepts that tend to inhibit the growth of microorganisms on foods, maintaining its safety and freshness. Also, it is an alternative to non-thermal process that prevents the growth of heat-resistant microorganisms and their spores (Barbiroli et al. 2012; Hatice et al. 2019). On grounds of its potential to provide safety and quality benefits, film based antimicrobial packaging is drawing attention from researchers (Amin et al. 2018). Antimicrobial agents when incorporated into packaging materials inhibit the growth of microorganisms at the surface which is much prone to spoilage and contamination. This approach efficiently lowers the need for adding large quantities of antimicrobials into the bulk of foods (Koutsoumanis and Skandamis 2013).

Earlier researches suggest that a controlled release from the packaging film to the food surface is more advantageous over dipping and spraying (Buonocore et al. 2003; Broek et al. 2015). In this approach, natural or chemical antimicrobial agents incorporated into the packaging system/polymer ensure active packaging, thus limiting/preventing the growth of microbes by reducing their growth rate or extending their lag phase. Figure 15.1 illustrates seven types of antimicrobial food packaging system that are currently in vogue. Different types of ingredients along with polymeric materials are generally incorporated into the packaging system for development of biofilm based active packaging system. This extends shelf life besides improving the safety of the product. Due to the increase in demand for preservative-free products and less processed food, low levels of preservatives should be applied to packaging as they come in contact with food or other natural preservatives. Development of antimicrobial food packaging materials by incorporating natural antimicrobial agents into a polymeric material is a novel approach in food processing/packing industry. A thorough study of the existing works of literature specifies that natural antimicrobial agents such as spice volatile oils (black pepper, sage, thyme, rosemary, garlic, etc.), plant extracts (grape seed extract, olive leaf extract, etc.), organic acids, viz. citric acid, acetic acid, lactic acid, etc.), and bacteriocins (lysozyme, colicin, nisin, pediocin, etc.) are used to produce antimicrobial packaging materials (Fernandez-Pan et al. 2014; Marta et al. 2014; Rabin and Salam 2014).

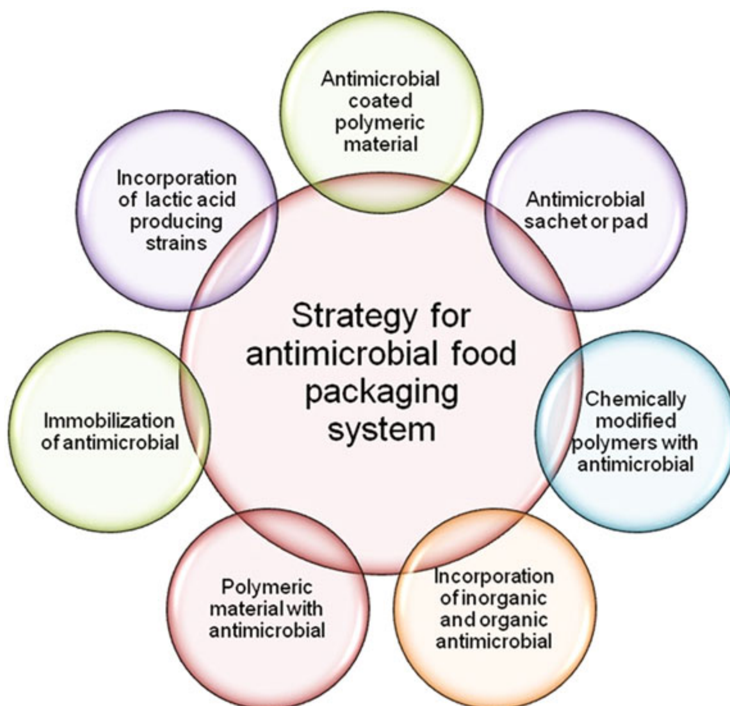

Fig. 15.1 Development of the antimicrobial food packaging system

15.2 Biodegradable Active Packaging System: Concepts and Commercial Applications

Chemically synthesized polymers have a greater demand as packaging materials on account of their low permeability values, excellent mechanical properties, and low cost. However safe environmental disposal of these materials is a major drawback. Environmental problems are due to non-renewable raw materials used for the development of the packaging system and subsequent accumulation of such non-biodegradable packaging elements in the environment. To overcome this challenge, research is focused on developing and manufacturing alternative packaging materials to chemical synthetics, considering the mechanical and permeability properties with economic feasibility. These biodegradable packaging materials that originate from completely renewable natural polymers have the ability to lessen environmental pollution and create space in the markets for agricultural products. But, incorporation of the antimicrobials into the packaging materials appends to the complications and manufacturing cost of the films. However, the costs of the film could anyway decrease in certain cases where replacement of expensive

antimicrobials with the cheaper ones would still account to higher antimicrobial activity (Chana-Thaworn et al. 2011).

Presently, the production and marketing of eco-friendly cum biodegradable packaging materials have higher scopes in developing countries. However, in many cases the petroleum-derived polymers such as polystyrene and polyethylene still rule the packaging industry, posing severe threats to global concerns (Bitencourt et al. 2014). This leads to problems concerned with performance, processing, and costing, all of which are still big questions for these non-renewable materials. In this context, various types of materials used for packaging of food products have been reviewed earlier based on the biopolymer that includes both coatings, edible films along with packaging materials (Aloui et al. 2014). Tables 15.1 and 15.2 summarize reported examples of different types of biodegradable matrices with additives. Rationally, the use of either biodegradable and renewable materials composed of polysaccharides, proteins, lipids, polyesters or even a combination of all can turn out to be an effective solution to these problems.

In addition to that, some useful additives such as antioxidants, colorants, and antimicrobial agents can provide some functional properties to the packaging materials in order to inhibit or delay microbial or chemical spoilage of packaged food items.

15.2.1 Moisture Absorbers

Food products are required to control free water in order to enhance their shelf life. The presence of seeping liquids such as blood or other fluids in fish and meat products lessens the quality of the food items. An excess amount of water inside the packaged foods favors the growth of microbes, causing softening of food products like cookies and biscuit as well as hardening freeze-dried coffee and milk powder. On the other hand, excessive water loss may also promote the oxidation of fat. Thus, organic acids have been incorporated into the absorbent pads to inhibit the growth of microorganisms in these nutrient-rich foods. The entire mechanism is based upon the absorption process, which tends to either remove excess water or control the relative humidity in the headspace depending on the requirement. This demands the use of highly hygroscopic and dehydrating agents such as polypropylene glycol, cellulose fibers, carbohydrates, minerals, polyacrylate salts, molecular sieves, silica gel, and calcium oxide, etc. The size and weight of food, as well as the initial water activity of the absorber, absolutely determine the type of moisture absorber to be selected. Moreover, factors like humidity and temperature of the storage foods, the transmission of water vapor from the packaged foods, and sensitivity of food towards the moisture play essential roles (Liz et al. 2013).

Table 15.1 The reported examples of different types of biodegradable matrix with additives against some microorganisms

Types of biobased matrix	Antimicrobial agent	Target microorganisms	Reference
Hydroxypropyl methyl cellulose	Extract of kiam wood (*Cotyleobium lanceotatum*)	*S. aureus*, *L. monocytogenes*, and *E. coli* O175:H7	Aloui et al. (2014)
Gelatin from skin	Bergamot and lemongrass essential oils	*L. monocytogenes, E. coli, S. aureus*, and *S. Typhimurium*	Ahmad et al. (2012)
Whey protein	Lactic and propionic acids, chitooligosaccharides, and natamycin	*S.* aureus, *Y. lipolytica*, and *E. coli*	Ramos et al. (2012)
Wheat gluten	Potassium sorbate	*Fusarium incarnatum* and *Aspergillus niger*	Türe et al. (2012)
Soy protein	Nisin, EDTA, and grape seed extract	*L.* monocytogenes, S. *typhimurium*, and *E. coli* O175:H7	Sivarooban et al. (2008)
Carboxy methyl cellulose	Potassium sorbate	*Aspergillus parasiticus* and *Aspergillus flavus*	Sayanjali et al. (2011)
Fish skin gelatin and egg white	Clove essential oil	*E. faecium, V. parahaemolyticus, C. perfringens,* and *P. aeruginosa*	Giménez et al. (2012)
Triticale protein	Oregano essential oil	*S. aureus, P. aeruginosa*, and *E. coli*	Aguirre et al. (2013)
Sunflower protein	Clove essential oils	*Clostridium perfringens*, *E. faecium*, *B. coagulans*, *B. cereus*, *V. parahaemolyticus*, *P. fluorescens*, and *A. niger*	Sánchez-González et al. (2013)
Pea protein isolate, hydroxylpropyl methyl cellulose, methyl cellulose, or sodium caseinate	Bacteriocins	*L. innocua*	Beshkova and Frengova (2012)

15.2.2 Antimicrobial Packaging

During the manufacturing process, microbiological contaminations are likely to occur when inadequate food processing or under-processing occurs. However, some of these methods are not feasible in case of foodstuffs like fresh meat, fresh fish and seafood. Some of the agents like carbon dioxide, ethanol, antibiotics, chlorine dioxide, organic acids, essential oils, and spices, etc., having antimicrobial properties have been investigated to inhibit the growth of microbes that can deteriorate foodstuffs (Campillo et al. 2009). Packaging systems like essential oils, carbon

Table 15.2 Some reported biopolymer with natural antimicrobial additives used in the development of film based packaging system

Biopolymers		Antimicrobial agents	Type of food	References
Cellulose and derivatives	Cellulose	Pediocin	Meat	Rodriguez-Lafuente et al. (2010)
	HPMC	Ethanol/citric acid/sorbic acid	Tomato	Rodriguez-Lafuente et al. (2010)
	Cellulose based paper	Nisin/Lacticin 3147	Cheese/ham	Johnson et al. (2018)
	Alginate	Nisin	Beef	Ishfaq et al. (2017)
		Nisin	MRS broth/skim milk	Ferhat et al. (2017)
		Nisin	Poultry	Natrajan and Sheldon (2000)
	Chitosan	Sodium benzoate/potassium sorbate	Culture media	Pastor et al. (2010)
		Acetic/propionic acid	Meat	Ouattara et al. (2000)
	Carrageenan	Chlortetracycline/oxytetracycline	Poultry	Natrajan and Sheldon (2000)
Proteins	Wheat gluten	Sorbic acid	Ethanol-water	Lim et al. (2010)
	Corn zein	Lysozyme/nisin	Culture media	Gomez-Estaca et al. (2010)
	Soy protein	Lysozyme/nisin	Culture media	Padgett et al. (2000)
	Whey protein	Potassium sorbate	Water-glycerol	Ozdemir and Floros (2001)
		p-Aminobenzoic/sorbic acids	Culture medium	Ozdemir and Floros (2001)
Polysaccharide	Starch and derivatives	Potassium sorbate	Straw berry	Perdones et al. (2012)

dioxide, plant extracts, allyl isothiocyanate also release volatile antimicrobials (Echegoyen and Nerín 2015). Chlorine dioxide existing in solid, liquid, gaseous has been proven to be effective for inhibiting the growth of various fungi, bacteria, and some viruses. It has potential applications in fish, poultry, meat, confectionery-baked foods, and dairy products (Yuyue et al. 2017). SO_2 has been used for controlling the decomposition of grapes, has better effects over the combination of gamma-radiation and heat treatment. The main drawback in case of SO_2 is bleaching grape-skin and that some of it may remain on the grapes (Yuyue et al. 2017). These residues leave a strong characteristic odor from the grapes that lead to product-rejection by consumers.CO_2 possesses a major role in the packaging atmosphere for inhibiting the growth of microorganisms and dropping the respiration rate of fruits and vegetables. CO_2 has to release continuously into the package for maintaining the

favorable concentration in most packaging films. It was found that CO_2 has permeability between 3 and 5 times as that of oxygen. Meat products require high levels of CO_2 (10–80%) to suppress the microbial growth over the product-surface, thereby extending their shelf life (Zoran et al. 2015; Toorn et al. 2017). Carbon dioxide has advantages of acting on all the food inside the package without any contact with the food products and package whereas changes in the color of meat and blanching of vegetables are some of the disadvantages.

15.2.3 Carbon Dioxide Emitters

Sachets and absorbent pads are used as CO_2 emitters for preserving the muscle-based foods. The packaging of salmon (Marta et al. 2017) and cod fillets (Yingchun et al. 2016) has been tested with carbon dioxide emitters to obtain enhanced shelf life using vacuum packaging. CO_2 emitters lessen a headspace of packaging container by limiting the supply of gas to food products ratio compared to optimally modified atmosphere packages (MAP). This indicates improvement in the activity of MAP-system without compromising in quality and shelf life. In recent literature, applications of carbon dioxide emitters and MAP are found to extend the product-life by avoiding the collapse of packages (Holck et al. 2014). The market for CO_2 emitters is likely to grow and develop towards further development of films incorporating the functionality of CO_2 emitters (Day 2008). A recent application has been studied on the applications of the active packaging system for controlling the quality of meat products applied as ready-to-eat (Chen and Brody 2013).

15.2.4 Oxygen Scavengers

Several types of scavengers for O_2 molecules have been utilized for eliminating residual O_2. It can extend the shelf life of muscle-based foods. The most adaptable scavengers of O_2 are based upon the oxidation of iron powder. The organic-type scavengers like catechol, ascorbic acid, and polyunsaturated fatty acids have been thoroughly studied in place of metal-based scavengers that have been incorporated to polymer blends of packaging substrate (Lee and Ko 2014).

Oxygen scavengers with the inclusion of microbes were studied as an effective alternative for chemical scavengers, on being advantageous regarding consumer perception and sustainability. Recently, active packaging with enzyme embedded barrier coatings has been studied in food applications (Järnström et al. 2013). The developed active coatings were found to serve successfully to avoid the oxidation and rancidity reactions in packed foods stored in chilled conditions. Incorporation of functional groups, which is unsaturated in nature provides oxygen absorption capacity, can enhance the oxygen barrier behavior of polymer films (Nydia et al. 2017). UV light triggers the auto-oxidation reaction in the polymer with the help of a metal-based catalyst. Development of a system for oxygen scavenger with a natural free

radical scavenger (for example, α-tocopherol) and a transition metal has done successfully (Kirschweng et al. 2017).

Another study indicated that nanoparticles loaded with α-tocopherol and iron chloride added to the gelatin films constituted the oxygen scavenging capacity (Byun et al. 2012). The inclusion complex showing stability in terms of its chemical-thermal stability is released. The nanocrystalline based on titania under UV radiation showed photocatalytic activity that has gained specific attention. An ascorbyl palmitate-β-cyclodextrin inclusion complex was found to be useful as an effective oxygen scavenger (Byun and Whiteside 2012). Titania nanoparticles were added to different polymers to successfully develop oxygen scavenger films (Jong-Whan et al. 2013). Organic substances can be oxidized O_2 consumption and CO_2 production due to the light-induced catalytic activity of nanocrystalline based on TiO_2 on the polymer surfaces. Photocatalytic titanium films have potential as antimicrobial packaging system as they are known for microbes-inactivation (Lee and Ko 2014).

15.2.5 Antioxidant Packaging

Fat oxidation is one of the vital processes responsible for food spoilage due to microbial growth. On the other hand, lipids oxidation in foods reduces their shelf life because of variations in taste and aroma. It also reduces the functionality, textures, and nutritional quality of the muscle foods and reduces the nutritional quality of the food products (Pereira de et al. 2010; Busolo and Lagaron 2012).

The application of oxygen scavengers and antioxidants packaging material can prevent the oxidation of food. The oxidation process which affects the food quality is thereby prevented or slowed down with such a packaging (Joaquín et al. 2014). Natural oxidants like Vitamin E and the extract rich in phenolic compounds (clove, oregano, cinnamon, ginger, etc.) are the additives that are gathering regard (Jeannine et al. 2018). Antimicrobial and antioxidant properties are exhibited by spices containing phenolic groups like flavonoids and various phenolic acids (Maria and Jose 2012). Yet, the major initiators of oxidation are the radicals (mainly hydroxyl, oxo, and superoxide) produced through the oxidation process. Rapid elimination of radicals on their formation itself can, therefore, prevent oxidation. Further oxidation is also prevented by some natural compounds that trap the radicals and react efficiently with them. The sole presence of a radical scavenger is far effective over high barrier or vacuum packaging materials to protect food against oxidation in such a case. The stability of myoglobin and the fresh meat against oxidation can be improved by the application of antioxidant-based active film used in the preservation of fresh meat (Silvia et al. 2017). An increase in fresh odor and improved oxidative stability has been observed in lamb steaks using an active film based on rosemary and oregano (Yingchun et al. 2016). Lipid oxidation in milk powder can be delayed through α-tocopherol migration from an active packaging film (fabricated with the high-density polyethylene, ethylene vinyl alcohol, and α-tocopherol) (Silvia et al. 2017). Apple slices have been covered with cellulosic films incorporated with cysteine and sulfite to obtain brighter apples with less browning (Magnea et al.

2014). Diffusion and evaporation of antioxidant substrate from the surface of the film lead to decreasing its contents during storage through the film. Addition of an extra layer of the film having low permeability to antioxidants (Elahe et al. 2018) or the use of cyclodextrins (Atul et al. 2017) can prevent this decrease in the concentration of antioxidants applied for fresh meat, nuts, butter, oil, fruits bakery products.

15.2.6 Bioactive Edible Packaging Films

Recent days extensive research works have been conducted to develop biodegradable and edible packaging material from natural polymers to avoid the problems caused by the synthetic plastics. These biodegradable packaging materials can remediate the environmental problems and growing health concerns. Basically, these kinds of films are prepared from fruits extracts, juice, pulp, and vegetable purees due to its edible property, easy collection, and facile processing. Utilization of agriculture by-products like fruit peels and vegetable skins has drawn the attention of the researchers to prepare this edible film, and this can address the sustainability of environment and resource re-cycling process. The phenolic compounds obtained from the phytochemicals play a major role as the additives in the edible package. These can even improve the functional and physical properties of the film through cross-linking within the protein or polysaccharides based (Nie et al. 2015).

The tea polyphenol was used as natural antimicrobial agents in various food industries considering its biocompatibility, nontoxic, and low cost by some researchers (Shao et al. 2018). The edible packaging film was developed by incorporating the active ingredients of tea polyphenol into protein, polysaccharides, gelatin, and starch, etc. (Li et al. 2012; Liu et al. 2015; Dou et al. 2018). The shelf life and quality of oil was maintained by developing the active film made from gelatin and impregnated with tea polyphenol by retarding the lipid oxidation (Volpe et al. 2017).

15.3 Factors to Be Considered for Commercialization of the Active Packaging System

While designing and modeling antimicrobial film or packaging system for food preservation, there are several factors to be taken into consideration. Also, the choice of each substrate in conjunction with antimicrobial substance plays a vital role in the development of antimicrobial packaging system. With varying types of foods and nature of the antimicrobial components, physico-mechanical activities of package might change accordingly. Various factors responsible for development of active packaging system have been illustrated in Fig. 15.2.

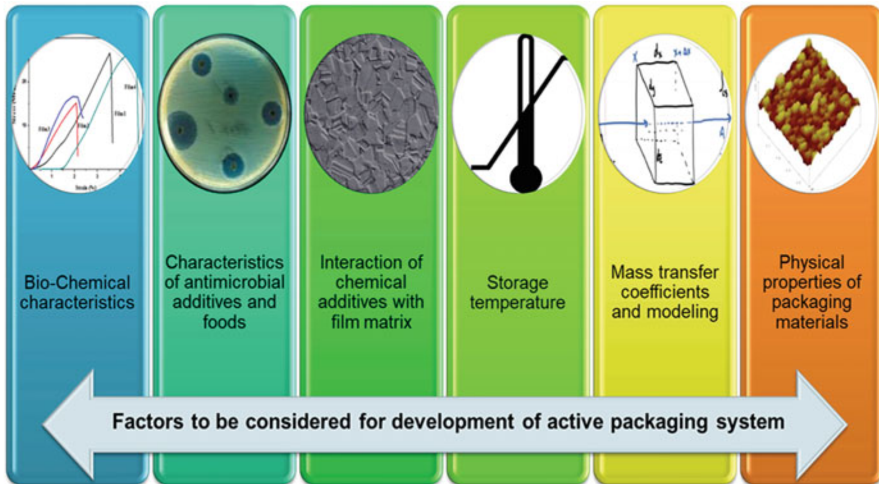

Fig. 15.2 Various factors responsible for development of active packaging system

15.3.1 Biochemical Characteristics of Films

As per earlier studies, the potency of an incorporated antimicrobial agent might sink throughout film fabrication, distribution, and storage (Mila and Carmen 2013). The cutting pressures and forces within the processing conditions in addition to the heat labile nature of element throughout extrusion boldly restrict the selection of antimicrobial (Agustín and Cecilia 2013). A mixture of low-density polyethylene (LDPE) resin and potassium sorbate powder help in the synthesis of a master batch. The heat decomposition of these pellets may be inhibited by the addition of LDPE resin (Amalini et al. 2018). Additionally, quantitative characterization of all other operations like lamination and drying in with the adhesive chemicals ought to be done. In some cases, during storage a good number of the volatile antimicrobial compounds could also be lost. Most of these parameters must be assessed.

15.3.2 Characteristics of Antimicrobial Substances and Foods

The effectiveness of antimicrobial substances and their release is considerably affected by food elements. The growth of microbes can be mathematically modeled by a typical study on the mechanism and dynamics of growth inhibition. Possibilities of alteration of the activity of antimicrobial substances by the physicochemical characteristics of the food cannot be eliminated. It is quite familiar that the growth rate of target microbes and the degree of ionization of the prime active chemicals are greatly affected by the pH of a product (Amalini et al. 2018). For example, polyethylene film containing carboxylic acid compound was reported to have more ability to inhibit molds at low pH levels. Indefinite quantity of oxygen will

be exploited by the aerobic microorganisms for their growth. The water activity of food can further influence the antimicrobial activity and chemical stability of incorporated active compounds considering the fact of every food having its own microflora. The rate of diffusion of potassium sorbate in MC/HPMC film containing saturated fatty acids was found to be high at higher values of free water. With respect to the contaminating microbe, designing of the release kinetics of antimicrobial agents should be done in a way that keeps up the concentration on top of the essential inhibitory concentration (Phakawat et al. 2015).

15.3.3 Interactions of Chemical Additives with Film Matrix

The polarity and mass of additive has to be considered necessarily during incorporation of additives into a polymer. Additives with high molecular weight and low polarity have extra compatibility with the non-polar LDPE. The diffusion rates of different additives within the polymer depend upon their ionic charge, molecular weight, and solubility. Recently, the diffusion of antioxidant, sodium ascorbate, and potassium sorbate in calcium-alginate films at 8 °C, 15 °C, and 23 °C was analyzed and experimented by some researchers. They found that antioxidant activity is the highest with sodium ascorbate and lowest diffusion rates at all the studied temperatures (Phakawat et al. 2015).

15.3.4 Storage Temperature

The antimicrobial activity of chemical preservatives may also be affected by the storage temperature. Usually, increase in storage temperature will result in high diffusion rates within the polymer leading to acceleration of active agent's migration within the film and deterioration of protective action of antimicrobial films (Elahe et al. 2018). The impact of temperature conditions on the antimicrobial activity of active compounds, their rate of diffusion, and the concentration within the film throughout production should be predicted in a way that sufficient temperature is maintained effectively throughout the product's life (Volpe et al. 2017). Few demonstrations by certain researchers state that low-density polyethylene (LDPE) containing low amounts of carboxylic acid anhydrides can be effective both at very low and high temperature (Kenneth 2017).

15.3.5 Mass Transfer Coefficients

Diffusion stands to be the most elementary system in facilitating release of active ingredient from packaging material into the food system. The advantages of using a multilayer packaging are that the antimicrobials are present in an additional skinny layer, their migration and release being controlled by the film density. It is also very important to manage the rate of migration of active substances from the food

package. The migration of active substances through the packaging systems composed of multi layers and can be described by a mass transfer model. The diffusion method may enable prediction of the release profile of antimicrobial agent and so the time through which the agent remains higher than the essential effectiveness concentration, by means of mathematical modeling. A semi-infinite model with a larger volume of food component, consisting of packaging element having a finite thickness which leads to infinite volume of the food element might be practical as compared to the packaging material (Lecoq et al. 2016; Ye 2016). The boundary conditions to be employed in mass transfer modeling are identifiable.

15.3.6 Physical Properties of Packaging Materials

The physical properties, processing, or mechanical aspects of the packaging material might be affected by the antimicrobial agents. On addition of the active agents to the heterogenous formulations, the performance of the packaging materials has to be retained (Jian-Hua et al. 2014). It was found in some studies that even on enhancing the concentration of benzoic anhydride from 0.5% to 1.0%, no variations in opacity and strength of LDPE film could be observed (Dobias et al. 2000). There were reports of similar results on plant extracts like propolis and clove. LDPE film coated with MC/HPMC exhibited a negative effect on the heat-seal efficiency due to the presence of nisin in it (Xiaowei et al. 2012).

15.4 Technological Applications in Active Food Packaging System

15.4.1 Application of Antioxidant Agent with Edible Films for Fruits and Vegetable Preservation

Various commercial applications of antioxidant-based packaging system for fruits and vegetables have been summarized in Table 15.3.

15.4.2 Application of Antioxidant Agent with Edible Films for Meat and Fish Preservation

Freshly caught fish spoils very quickly. With time spoilage rate accelerates. Practicing hygienic slaughter and handling of carcass help improve shelf life. Significant amount of spoilage in fish and meat is caused by bacteria. Autolytic spoilage due to fat oxidation and enzymes is also observed. Generally, tinned steel is used to store fish and meat products. Currently, antioxidant-based packaging materials are being used to preserve the products. Various commercial applications of antioxidant-based packaging system for fish and meat products have been summarized in Table 15.4.

Table 15.3 Antioxidant agent with edible films for fruits and vegetable preservation for commercial applications

Film or coating	Antioxidant compound	Application	Analyses	References
Chitosan coatings	Garlic, cranberry, carvacrol rosemary, onion	Squash butternut	Polyphenol oxidase and peroxidase activities	Volpe et al. (2017)
Whey protein concentrate and beeswax	Ascorbic acid, cysteine, and 4-hexylresorcinol (4-hexyl)	Apple	Weight loss, color, sensory evaluation	Kenneth (2017)
Methylcellulose polyethylene glycol and stearic acid in 3.0 g:1 mL ratio	Ascorbic acid, citric acid	Apricots and green peppers	Water loss, vitamin C	Lecoq etal. (2016)
Gellan and alginate with glycerol	Ascorbic acid	Papaya	Respiration rate, water loss, firmness, content of ascorbic acid, and production of ethylene	Jian-Hua et al. (2014), Ye (2016)

Table 15.4 Antioxidant agent with edible films for meat and fish preservation for commercial applications

Polymeric materials	Antioxidant compound	Application	Analyses	Reference
Milk protein-based film	Oregano and/or pimento essential oils	Beef muscle	TBA	Kenneth (2017)
Gelatin-based films with chitosan	Rosemary or oregano essential oils	Cold-smoked sardine	TBA, total phenol, FRAP method	Dobias et al. (2000)
Soy protein	Ferulic acid	Lard	PV	Garcia et al. (2004)
Alginate and glycerol coating	Sodium ascorbate and citric acid	Buffalo meat patties	TBA, tyrosine value, sensory quality	Ayranci and Tunc (2004)
Chitosan coatings	Fish oil, vitamin E	Lingcod fillets	TBA	Lecoq et al. (2016)

15.4.3 Polymer Nanotechnology in Packaging

Over 30,000 various natural and synthetic polymers are known today and it is important to understand their effects on human health before utilizing them as packaging systems. Bio-nanofibers such as chitosan, cellulose, and collagen have superior properties compared to traditional polymers—high specific surface area per unit of weight, excellent mechanical properties, and are light. These properties make them ideal, either as packaging materials or as additives to be integrated into polymer matrix by injection to improve function (Zhongxiang et al. 2017). New innovations

in nanotechnology for food preservation include active packaging involving the combination of antimicrobial substances with food packaging materials. This majorly includes incorporation of antibacterial nanoparticles into polymer films. It was observed that an intensive contact between food product and packaging material is necessary in the case of both migrating and non-migrating antimicrobial materials. Hence potential food applications include vacuum or skin-packaged products, e.g. vacuum-packaged meat, fish, poultry, or cheese. Nanocomposite materials reportedly show greater barrier properties, mechanical strength, and temperature resistance as opposed to traditional composites. Fillers like nanoclays, carbon nanotubes, kaolinite, and graphene nanosheets boost shelf life by acting against the diffusion of gases and flavor-giving volatiles (Attrey 2017; Elisa et al. 2018). Among the materials that received considerable attention for their sustainability are cellulose and polylactic acid (PLA) because they are biodegradable materials with suitable mechanical and optical properties. PLA, additionally, offers the advantages of easy production (its monomer lactic acid is a fermentation product of carbonaceous feedstock) and biocompatible disposal compared to traditional petro-based polymers. It is also possible to integrate antimicrobials with carriers like plastics, paper-based materials, textile fibrils, and packaging materials. Fluorescent nanoparticles integrated with antibodies help in identifying chemical and food borne pathogens. In order to successfully employ polymer nanotechnology in food technology it is important to consider the complete lifecycle of the material which helps in understanding its holistic impact in all stages of production and on all forms of life and environment. Life cycle assessment (LCA) studies help in monitoring the said impacts right from raw materials in production to usage until disposal. They also help in optimizing material and energy recovery for sustainability.

15.5 Recent Patents for Active Packaging System

For search of information related to active food packaging system, different web-resources have been used, such as Google patents, The Web of Knowledge, and WIPO (World Intellectual Property Organization). In order to analyze the application of active packaging in food with respect to issued patents, number of patents evolved from January 2009 to April 2019 was retrieved from the PATENTSCOPE database under WIPO. The search was performed using “FP:(Active Packaging of food)” as a keyword. Six hundred and sixty-one number of the patents on the application of active packaging in food were published in the last 10 years. For all the obtained data, various aspects like: title, abstract, assignee country, number and date of deposit, etc., were analyzed. It was found that, China has contributed maximum number of patents (227) in this field followed by Russian Federation (114) till date as shown in Fig. 15.3.

The number of applications increased in 2012, peaking in 2017, signaling a potential increase in product and process innovation in a global context. After 2017, there is a slight decrease and constancy was observed in the following years. 2017 was the year with the highest number of patents granted (Fig. 15.4).

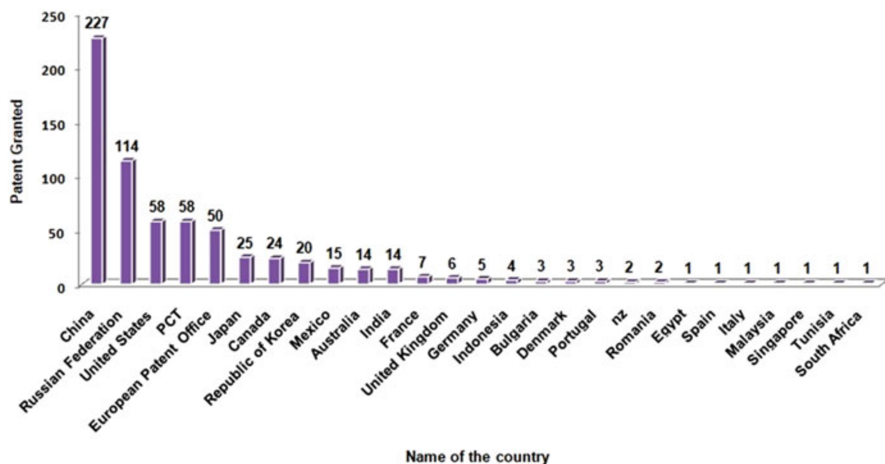

Fig. 15.3 Patents granted worldwide in the topic of active packaging system of food obtained from the PATENTSCOPE database under WIPO as on year 2019 (Source of Data: https://patentscope.wipo.int/search/en/result.jsf)

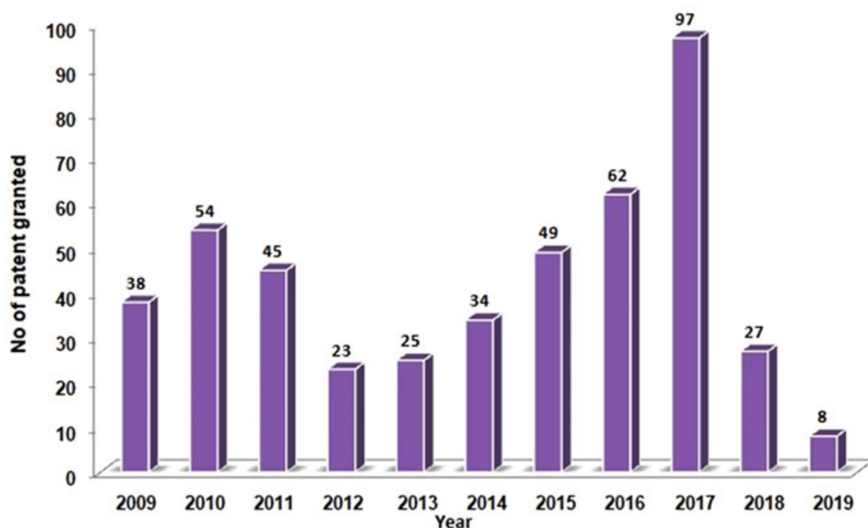

Fig. 15.4 Year wise patent granted in the area of active food packaging system obtained from the PATENTSCOPE database under WIPO as on year 2019 (Source of Data: https://patentscope.wipo.int/search/en/result.jsf)

Active food packaging technologies are evidently growing in size, to extent that they are best described as an "emerging technology." It was found by the key IPC (International Patent Classification) class level for the largest technology groups. The IPC A23L contributes maximum, i.e. 32%, which denotes human necessities

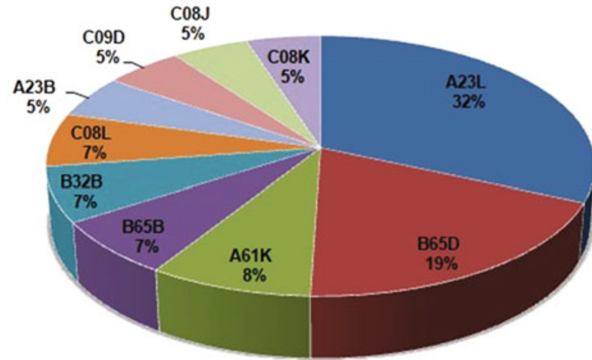

Fig. 15.5 Active packaging system with respect to IPC (International Patent Classification) contribution obtained from the PATENTSCOPE database under WIPO as on year 2019 (Source of Data: https://patentscope.wipo.int/search/en/result.jsf)

(Food, food stuffs, and non-alcoholic beverages). IPC B65D contributes 18%, denotes performing operations, (Containers for Storage or Transport of Articles or Materials). Development of food packaging bags, barrels, bottles, boxes, cans, cartons. The IPC C08L contributes 7%, applicable for organic macromolecular compounds and their preparation or chemical working-up in the development of active packaging (Fig. 15.5).

15.6 Intelligent Packaging System

15.6.1 Radio Frequency Identification (RFID) System

RFID technology cannot be classified either as a sensor or indicator whereas it constitutes a separate information-based electronic device to form an intelligent package device. RFID transmits accurate and real-time information using tags attached to assets (containers, pallets, cattle, etc.). It is an automatic identification system with a group including bar-codes. Hence, it is offering a number of probable aids in meat production and distribution. This aid comprises inventory management, traceability, security, and safety (Min and Peichong 2012). RFID technology also plays a major role in prevention of product recalls (Nora et al. 2012). Though the application of RFID technology to packaging is quite a latest development, it has been accessible for about 40 years (Lindsey et al. 2014). Shipping to suppliers, receipt by distribution centers (DCs), and put-away processes with retailers can be improved through the access of RFID technology in retail industry (Fabien et al. 2017). There have been reports regarding a fruit warehouse employing the RFID-based traceability system (Lindsey and Ning 2013). In addition to that, RFID technology was applied to the British livestock industry to explore the significance of the traceability system as well as closely study its potential costs and utility (Yong-Shin et al. 2012). Apart from these, improvement in replenishment productivity and reduction of stock loss in the supply chain of short shelf life products can be achieved by adopting RFID technology. RFID technology is accessible through the complete supply chain for retail-foods industry which consists of tighter

management, control of the supply chain, reduced shrinkage and labor costs, improved customer service, and compliance with traceability protocols and food safety regulations (Rahul et al. 2017).

15.6.2 Time–Temperature Indication System

Time and Temperature Indicators (TTIs) on account of their simplicity, affordability, low cost, and efficiency have been largely utilized (Zabala et al. 2015). Development of various TTI trade has occurred on the enzymatic base and both polymeric and biological reactions. Kinetic study with modeling of loss ratios, food quality and response poses as a prerequisite for the productive implementation of a control system based TTI (Tsironi et al. 2011). It is essential to track the changes in parameters like time and temperature from production to consumer in order to ensure the quality and safety of food products that require optimal temperature (Wu et al. 2015). TTI can be stored in the form of a small sticker, in transport or individual containers. The exposure of food to a different recommended temperature will reflect an irreversible chemical change. In cases where cold storage is a critical control point during transport and distribution, TTI delivers vital contributions for the safety and quality of chilled/frozen food (Kuswandi et al. 2012). TTI indicates of products being heated above or cooled below a critical temperature, thus warning the consumers about the chances of the survival of pathogenic microbes and protein denaturation processes. The pasteurization and sterilization processes can be analyzed by application of TTIs (Kim et al. 2013, 2016). A collective indication of the storage temperature to which the TTI is exposed, is given by the visible response. Depending on their response mechanism, partial history or full history indicators are the classifications of TTI. There is no response by partial history indicators until a temperature threshold has been exceeded, indicating the exposure of a product to a temperature that is sufficient to change its quality or safety. A continuous temperature-dependent response is given by Full history TTIs throughout the history of a product, thus constituting the prime focus of interest for research and commercial use (Chun et al. 2017). The chemical, enzymatic, or microbiological changes initiated by the response ought to be visible, irreversible, and temperature-dependent (Eun-Jung et al. 2014).

15.6.3 Gas Indication System

The activity of the food product, nature of the package, environmental conditions lead to changes in the gas composition in the package headspace. O_2 and CO_2 are utilized for monitoring the food quality, and as seal indicators (Chompoonoot et al. 2014; Tumwesigye et al. 2017). A change in the color is also detected due to chemical or enzymatic reactions occurred by O_2 or CO_2 indicators. A color change is indicated, if the concentration of the O_2 exceeds a predefined limit in a sealed food package. Redox dyes such as methylene blue, 2,6-dichoroindophenol, and N,N,N′,

N′-tetramethyl-p-phenylenediamine are used as O_2 indicators (Lina et al. 2017). Similarly changes in color also occurred when CO_2 concentration decreases to a specific level. It has found that visual indication for release of CO_2 is formed by calcium hydroxide. Indicator of CO_2 is caused by calcium hydroxide. The O_2 sense is an indicator for the products packed in modified atmosphere packaging (MAP) (Alessia et al. 2013; Chau and Keehoon 2013). However, such type of indictor requires anaerobic condition to be maintained as it deteriorates quickly in the presence of air. The application of indicator which changes its color in the presence of oxygen is one of the novel approaches. In some cases, the indicator has to be activated with UV light (Pradeep et al. 2012).

15.6.4 Pathogen Indication System

Detecting the presence of harmful microorganisms in food is a novel way to identify contamination before consumption. Food Sentinel System™ and Toxin Guard™ are the commercially available visual indicator system used in the packaged food. Here, food must not be consumed if the color has changed. The Food Sentinel System™ is a visual system that chromogenically indicates contamination in food packages. The underlying mechanism is that it detects the presence of harmful microorganisms through immune reactions that make the barcode unreadable. In many cases, an antibody specific pathogen (*Escherichia coli* 0157:H7, *Listeria monocytogenes*, *Salmonella*) is made fixed to a membrane that gives rise to a product of the barcode (Goldsmith et al. 1999). Another commercial indicator known as Toxin Guard™, which is used to detect the freshness and biochemical characteristic through the incorporation of monoclonal antibody in a polymeric base. It can detect other pathogens such as *Campylobacter* sp., *Salmonella* sp., and *Listeria* sp., *Escherichia coli*. Moreover, it can detect the metabolites produced through the bacterial degradation of the food product (Goldsmith et al. 1999).

15.7 Market and Legislative Considerations for Packaging System

Packaging sector is a diverse industry with each of its sectors having significant impact on global markets. There is a growing interest in consuming fresh products with extended shelf life and controlled quality and hence it is upon the manufacturers to provide safe packaging. This is the main challenge as well as the driving force to develop improved strategies and technology (Aaron and Erik 2017; Kalliopi et al. 2018). Packaging materials that serve as an adequate barrier to gases, UV protection, and extension of the storage period, transparency, and environmental performance are hence under great demand and are reportedly the future of food packaging (Farmer 2013; Lindh et al. 2016). This is supported by the fact that advanced packaging represents about 5% of the total value of the packaging market, of which 35% belongs to active systems. There are indications that soon there would

be a further increase in these numbers in the coming years, both in their market share as well as their patent grants.

Though the origin of such solutions was in Japan and the USA initially, they have now crept into the formerly restrictive Europe (Ghaani et al. 2016). The latter further indicates the growth in research interest in those particular problems in their markets (Lee et al. 2015). These trends also suggest that active materials must be brought to the market after proper and effective application and after their screening for meeting legislative requirements. The changes that these materials may undergo with time when in contact with food must also be considered (Realini and Marcos 2014).

15.8 Bioplastic: The Upcoming Food Packaging Material

The synthetic plastic used in the food packaged materials creates major problems like toxicity, decomposition, and its accumulation in the environment. In some cases, it induces the ecological impact very badly in case of landfills and also causes water contamination. The solution to avoid these problems associated with synthetic plastic would be the replacement of synthetic polymers by biodegradable polymers. The bioplastic can be prepared on the basis of three combinations as follows:

- Petroleum-based bioplastic: These are synthesized from petroleum resources. However, these are found to be biodegradable by nature at the end of their functionality.
- Mixed source-based (bio- and petro-) bioplastic: These are synthesized and developed with a combination of biobased and petroleum monomers.
- Renewable resource-based bioplastic: These are either synthesized naturally from plants and animals, or from renewable resources.

Various categories of bioplastics have been illustrated in Fig. 15.6. In the present days, bioplastic production contributes only one percent of 320 million tonnes of plastic production annually. As per the report given by European Bioplastics, global bioplastic production will be increased to 2.44 million tonnes in the year 2022 based upon the demand and revolution for environmental sustainability (Rukhsana et al. 2019).

Bioplastic based food packaging system and its commercial applications have been summarized in Table 15.5.

It was concluded that, PLA (polylactide) is mostly used in the market out of various biobased materials commercially. However, main applications for bioplastics are used for short shelf life products. Several researches on PLA based packaging proved that they could replace the conventional packaging system. In one study, the comparison of whole green peppers packed in a PLA based film and LDPE (low-density polyethylene) was investigated. There was no significant difference in color, hardness, and ascorbic acid concentration was observed (Koide and Shi 2007). It was also reported that PLA packaging is suitable for storage of fresh orange juice at 4 °C for 14 days. Moreover, the color changes, ascorbic acid

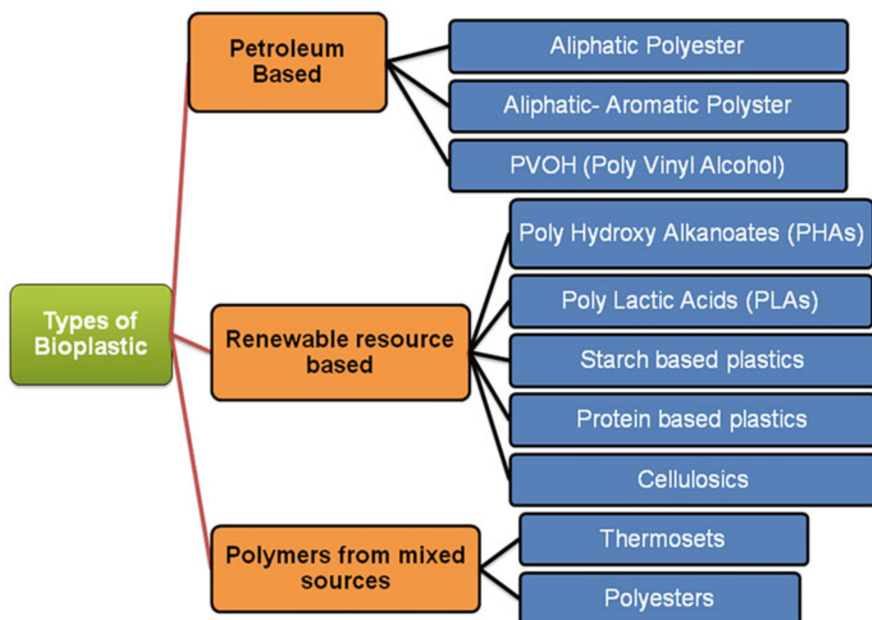

Fig. 15.6 Types of bioplastic used as food packaging material

Table 15.5 Bioplastic based food packaging and its commercial applications

Food types	Types of bioplastic	Company/industry	Reference
Yogurt	Jars made up of PLA	Stonyfield (Danone)	Peelman et al. (2013)
Frozen fries	Films based on PLA (bio-flex)	McCain	Rukhsana et al. (2019)
Freshly cut fruits, vegetables, salads, and bakery goods	PLA trays	Asda (retailer)	Rukhsana et al. (2019)
Coffee and tea	Cups coated with PLA	KLM	Peelman et al. (2013)
Butter	PLA based container	Cyclus (Brasil)	Rukhsana et al. (2019)
Fresh salads	PLA based bowls	McDonald's	Peelman et al. (2013)
Potato chips	PLA based bags	Snyder's of Hanover, PepsiCo's Frito-lay	Blakistone and Sand (2007)
Kiwi fruit	Cellulose film based trays	Walmart	Blakistone and Sand (2007)
Bread	PLA based paper bags	Delhaize	Peelman et al. (2013)
Organic pretzels	PLA bags	PepsiCo's	Blakistone and Sand (2007)

degradation were most effectively prevented by PLA as compared with other packaging materials (Haugaard et al. 2003). The effect of pasteurization on a meat salad packed in conventional (PE: Polyethylene, PP: polypropylene) and biobased packaging (PLA, PHB: polyhydroxybutyrate) material was investigated and PHB films were found to be suitable packaging materials for this purpose (Levkane et al. 2008). Different studies on cellulose based films also showed that they could be an alternative for packaging several food products.

However, there are some limitations like thermal instability, brittleness, difficult heat sealability, low melt strength, and high oxygen permeability restrict the use of PLA and PHB films for many food packaging applications. Starch and cellulose are hydrophilic by nature. Hence, packaging materials based on these materials have a low water vapor barrier, which leads to poor mechanical properties and limited long-term stability.

15.8.1 Roles of Microbe in the Remediation of Bioplastic Based Packaging Materials

The biodegradation and catabolism of bioplastics are responsible for more than 90 forms of microbes. Degradation of bioplastics by bacteria or fungal organisms is known by the presence of a clear zone surrounding development in a plate comprising the bioplastic as the sole source of carbon, accompanied by analysis of the diameter for the extension of biodegradation.

Destabilization of PHA bioplastic has been found using the PHA biodegradation verification technique by using scanning electron microscopy (Tachibana et al. 2013). Enzymes that may be whether intracellular or extracellular are accountable for bioplastic degradation. Depolymerases group of enzyme, which was derived from microorganisms, has been investigated and was found to play an important role in biodegradation of bioplastics (Chua et al. 2013).

The depolymerase enzyme produced from *Streptomyces thermoviolaceus* is responsible for the degradation of bioplastic (Chua et al. 2013). Certain microbial enzymes including lipase and estrase produced from *Alcaligenes faecalis* and *Comamonas acidovorans*, respectively, involved in bioplastic biodegradation (Trivedi et al. 2016). Soil and compost habitats have been extensively studied, and were found to contain a high number of bioplastic degrading microorganisms (Accinelli et al. 2012). Due to various higher organic content, agricultural soils were reported as an effective site for PLA degrading organisms (Penkhrue et al. 2015).

15.9 Future Prospects and Safety Issues

Antimicrobial packaging is an emerging technology that promises a new approach to food packaging. Despite its current restricted use due to legal issues related to additives, it does not cease to be an innovative viewpoint for the present and future

packaging trends. The need for versatility of packaging methods stems from the increasing demand for convenience, safety, and compatibility of food product packaging, combined with the need to create environment friendly solutions to problems. A lot of research is still underway before commercializing innovations. A thorough analysis of the chemical, microbiological, and physiological effects of such systems on food, its consumers, and the environment is the need of the hour. Thus far, strategies and model systems have been studied. Yet a lot of work is to be done on trials with actual food materials and their preservation (Vanderroost et al. 2014).

Potential food applications of antimicrobial films include fish, cheese, bakery product, meat, fruits, and vegetables (Pereira de Abreu et al. 2012; Dobrucka 2013). This is very important to identify the categories of food that will make benefited most from antimicrobial packaging materials. Future research into a mixture of naturally derived antimicrobial agents, bio preservatives, and biobased polymers can highlight a variety of antimicrobial packaging system considering the food safety, shelf life, and environmental friendliness is necessary (Noushin et al. 2017; Soares et al. 2017; Giuseppe et al. 2018). Also, additional analysis is required on utility of plant extracts so as to judge their antimicrobial activity considering its potential side effects in prepackaged foods (Dariusz and Barbara 2014; Zárate-Ramírez et al. 2014). Thus, analysis is required to work out whether or not natural plant extracts may act as each an antimicrobial agent and as an odor/flavor enhancer. Amendments to regulations also require toxicological testing before use (Ye-Chong et al. 2014).

15.10 Conclusion

Several reviews validate the effectiveness of antimicrobial agents to inhibit the growth of microorganisms when incorporated into the packaging materials. Further work has to be carried out in correspondence to the factors affecting inhibitory characteristics of the antimicrobial systems. Very less research has performed about the sensory effects of these additives on food, particularly with respect to plant extract additives that release pungent odors. Further study is also required regarding the effects of additives on the physical characteristics of the film. It should be remembered that, primary objective of active packaging system is to provide healthy and safe food with high quality control standard. In order to set a sustainable environment and prevent the possible disposal of packaging wastes in the environment, production of bioplastics gained a lot of attention due to their biodegradability. The research and development in the area of bioplastic based active packaging system would be helpful to enhance the shelf life of the food products in eco-friendly ways.

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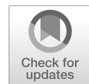

Biotransformation: Basics and Applied Perspectives

16

M. H. Yashavarddhan, Sania Bajaj, Sandeep Kumar Shukla, and Arpana Vibhuti

Abstract

Biotransformation is integral part of a living system. The system is built upon materials which are absorbed and transformed into the organic and inorganic building blocks and the unwanted or/and toxic materials are transformed and eliminated out of the system. These biotransformations are a part of multistep process containing specific enzymes and reactions catalyzed by them. Further these reactions and transformations are governed by biological factors, physiochemical properties, etc. of the system and materials which are taken in. Therefore, biotransformations can be used as technical strategy for pollution abatement, production of pharmaceutics, compounds especially for chemical and agricultural industry, etc. The current chapter delineates different phases of biotransformation process, the enzymes and their applications in industry and briefly sums up the current status of available research.

16.1 Introduction

The process by which a xenobiotic compound is transformed from one form to another within the living body is called biotransformation. This process can be completed by the involvement of an enzyme or multiple enzymes and/or organisms such as fungi, bacteria, etc.

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The Pharmacology and Experimental Therapeutics Department Glossary at Boston University School of Medicine propose the following definition:

Chemical alteration of an agent (drug) that occurs by virtue of the sojourn of the agent in a biological system (Pelikan 2004). This statement rightly explains and summarizes the true meaning of word Biotransformation.

In our day to day life we take in many compounds which are converted by our body into useful materials such as nutrients required for regular body functions. Due to metabolic activities, wastes are produced which need elimination. By virtue of biotransformation, the xenobiotic compounds entering the body and the body wastes are converted into less harmful substances that can be excreted out. Human body has an efficient biotransformation system for most of the xenobiotics and body wastes (Liska 1998). Hemoglobin, the oxygen-carrying iron–protein complex in red blood cells (RBCs) is one of the examples of body waste which should be eliminated after the RBCs complete their lifespan and the hemoglobin complex is broken into globin and heme. The heme gets fragmented into biliverdin, which is rapidly reduced to bilirubin, an orange-yellow pigment (Kuntz 2008). If the body is unable to excrete out the bilirubin via the liver due to a disease, medicine, or infection, it builds up in the body. As a result, the eyes and the skin may look yellow indicating the development of jaundice (Walker et al. 1990). The presence of high concentrations of bilirubin can cause irreversible brain injury in a newborn baby (Hansen and Bratlid 1986). However, liver break downs the lipophilic bilirubin molecule via biotransformation process into water-soluble hydrophilic metabolites which are converted into bile and eliminated through feces. Thus, the process of biotransformation is essential for survival of a living organism.

16.2 History of Biotransformation

Historically, most studied biotransformations were catalyzed by micro-organisms and a lot of research has been conducted on this subject in the 1970s. Due to expansion of science and technology, mechanisms and the probable agents of biotransformation could be explored in 1980s. With the introduction of enzyme technology, including enzyme and cell immobilization techniques, a much wider potential choice biocatalysts became available. During the 1980s there has been a much greater awareness that biological catalysts can also be used to transform reactants which have low solubility in aqueous media (Lilly 1992). Thus with the help of new technologies and knowledge, research shifted more towards searching for the mechanism at molecular level and exploring applications for handling the pollutants also. This makes it important to know about the process and its working in the organisms.

16.3 The Sites and Phases of Biotransformation Reaction

16.3.1 Sites for Biotransformation Reactions

Liver Predominantly, liver is the major and largest site in the body which contributes to both the pre-systemic and the systemic elimination of several xenobiotic compounds and the metabolism involved is called the hepatic metabolism. It has the largest concentration of enzymes required for transformation of compounds.

Intestinal Mucosa Cells The cells in the mucosal lining contain specific enzymes and microflora which convert the orally administered compounds and prepare them for complete conversion in the liver. The intestinal tract helps in the hepatic metabolism of the compounds as the blood from intestine flows directly into the liver via hepatic portal vein instead of going into general circulation (Pang and Kwan 1983; Liska 1998).

The other organs for extra-hepatic metabolism are kidneys and lungs which have only 10–30% liver's capacity. Skin, testes, and placenta have very low capacity for biotransformation (Vīna et al. 2013).

16.3.2 Different Phases of Biotransformation Process

Major contributions to the knowledge of metabolism and toxicology of drugs and xenobiotics were made by professor Richard Tecwyn William who is also known as founding father of drug metabolism. He not only studied the processes of transformation of several drugs but also classified these reactions and introduced the concept of phase I and phase II metabolic pathways in the second edition of his book "Detoxication mechanism: The metabolism of drugs and allied organic compounds" (Williams 1959). He suggested that in the first phase or phase I, oxidations, reductions, or hydrolysis or a combination of any of these three occurs, whereas in the second phase or phase II, it uses the metabolites of phase I and conjugates them with another molecule (Table 16.1). He also argues for making the distinction between phase I and phase II reaction. He suggested that the phase II conjugation reactions were always deactivating toxicity, whereas phase I reactions could either activate or deactivate depending on the compound (Williams 1959).

Table 16.1 Lists of major biotransformation reactions for xenobiotics broken into phase I and phase II reactions

Phase I	Phase II
Oxidation	Sulfate conjugation
Reduction	Glucuronide conjugation
Hydrolysis	Glutathione conjugation

After William's theory, many researchers proved his concept and end up developing the thought that phase I reactions must by necessity precede phase II. However, in some of the cases, the metabolic process can bypass all phase I chemical manipulations and undergo conjugation, i.e. phase II reaction directly (David et al. 2005).

16.3.2.1 Phase I Reactions

In general, phase I reactions are reactions which modify the chemical by adding a functional structure which is usually a small polar group which contains both positive (+ve) and negative (−ve) charges (Lech and Vodicnik 1985). The addition of a functional group permits the substance to fit into the enzymes of phase II reaction. The phase I reactions are further classified into:

1. Oxidation
2. Reduction
3. Hydrolysis

Oxidation

It is the most common reaction of phase I. It is correctly defined as a process where the substrate loses electrons. There are a number of reactions which have the ability to remove electrons from the substrate but because the addition of oxygen, or oxygenation, was the first discovered so it was named oxidation. However, most of the oxidation reactions do not involve oxygen (Lech and Vodicnik 1985; David et al. 2005).

Example Dehydrogenation is one of the examples of the oxidation reaction, which removes the hydrogen from the molecule. Most of the oxidizing reactions are explained by the name of the reaction or enzyme involved. Few of these oxidation reactions are as follows:

- Alcohol dehydrogenation
- Alkyl/acyclic hydroxylation
- Aromatic hydroxylation
- Deamination
- Desulfuration
- N-dealkylation
- N-hydroxylation
- N-oxidation
- O-dealkylation
- Sulfoxidation

The drugs which undergo oxidative metabolism include ropivacaine, paracetamol, omeprazole codeine, and phenothiazines.

Reduction

This is the second type of phase II reaction. The gain of an electron by the substrate in a chemical reaction is termed as reduction reaction. The reducing agent refers to the element that accepts electrons and in the reaction, one compound is oxidized and another compound is reduced. The reactions are most likely to occur in xenobiotics with low oxygen content. Reductions can take place across nitrogen–nitrogen double bonds, termed as azo reduction, or on nitro groups (NO_2). Commonly, the resultant amino compounds get oxidized to form toxic metabolites. In biological tissues, carbon tetrachloride can be reduced to free radicals which are very harmful to the body. So, the reduction reactions frequently result in inactivation of a xenobiotic rather than detoxification (Lech and Vodicnik 1985; David et al. 2005). An example of a reduction reaction in which the nitro group is reduced is illustrated in Fig. 16.1.

Most of the reduction reactions are explained by the name of the reaction or enzyme involved. Few of these reduction reactions are as follows:

- Azo reduction
- Dehalogenation
- Disulfide reduction
- Nitro reduction
- N-oxide reduction
- Sulfoxide reduction

Examples of drugs which undergo reduction include a prodrug called prednisone, which is reduced to the active glucocorticoid prednisolone; warfarin, an anticoagulant.

Hydrolysis

It is the final reaction of phase I. The hydrolysis reaction is breaking of toxicant into two fragments or smaller molecules when a water molecule is used to break the bond. The hydroxyl group (OH–) is attached into one fragment and the hydrogen atom is attached into the other fragment (Lech and Vodicnik 1985; David et al. 2005). These hydrolysis reactions mainly happen between an ion and water.

Example In a biotransformation reaction, the procaine compound produces two major smaller chemicals (Fig. 16.2).

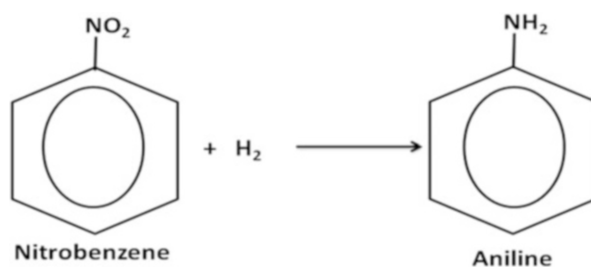

Fig. 16.1 Reduction reaction in which the nitro group is reduced (from NO_2 to NH_2)

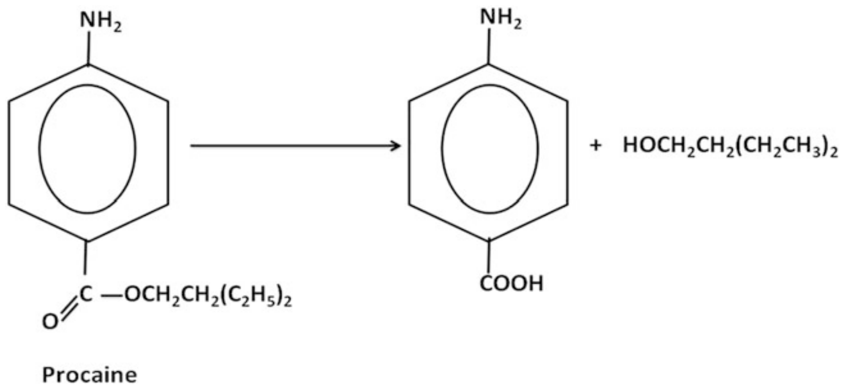

Fig. 16.2 Hydrolysis of procaine

Drugs which undergo hydrolysis reaction include prilocaine, an anesthetic agent; remifentanil, an analgesic drug.

In phase I biotransformation, toxicant converted into metabolites which are hydrophilic, or sufficiently ionized or to be either eliminated from the living body without further biotransformation or it can have the ability to convert the intermediate metabolites which were ready for phase II biotransformation process. In most of the cases, the intermediates forms due to phase I reaction may be very useful or it may be toxic than the parent (Lech and Vodicnik 1985).

16.3.2.2 Phase II Reactions

Phase II biotransformation reactions generally serve as a detoxifying step in metabolism. These reactions consist of those enzymatic reactions that conjugate the modified xenobiotic with another substance. Usually, the conjugated products are larger molecules than the substrate and polar or water-soluble in nature. These help in their rapid excretion from the body in bile or urine. The primary phase II reactions are as follows:

- Glucuronide conjugation
- Sulfate conjugation
- Acetylation
- Amino acid conjugation
- Glutathione conjugation
- Methylation

Some of the important phase II reactions were explained below.

Glucuronide Conjugation

The glucuronide conjugation is one of the most common and important types of phase II reactions. As the name suggests glucuronic acid molecule is used for conjugation in this reaction. The term was derived from glucose, which is the most common carbohydrate (sugar) and energy source for the cells inside the living body.

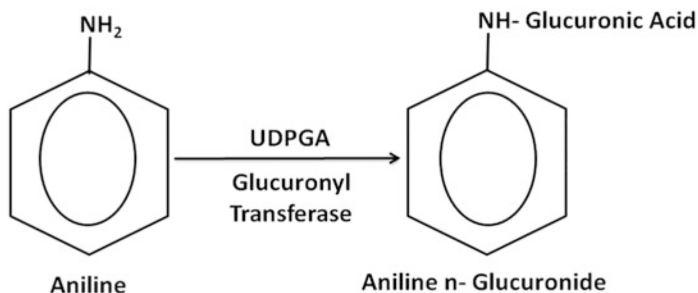

Fig. 16.3 Glucuronide conjugation of aniline

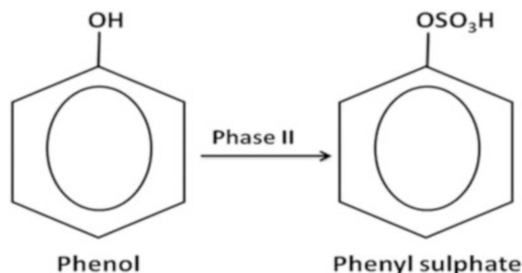

Fig. 16.4 Sulfate Conjugation of phenol

The substrate having nitrogen, oxygen, or sulfur bond is the site for the glucuronidation reactions and is applied to a wide array of xenobiotics as well as endogenous substances, such as thyroid hormones, steroid hormones, and bilirubin, etc. In glucuronidation reaction pathway, the conjugates xenobiotics are at a high capacity and the conjugation usually decreases the level of toxicity. However, there are some notable exceptions, for example, carcinogenic substances. These conjugates are generally hydrophilic in nature and are eliminated out by kidney or bile, depending upon the size of the conjugate (David et al. 2005; Jancova et al. 2010).One of the examples of glucuronide conjugation is conjugation of aniline to aniline *n*-glucuronide (Fig. 16.3).

Drugs which undergo glucuronidation are morphine, midazolam, etc.

Sulfate Conjugation

In phase II reaction, sulfate conjugation is another important reaction which occurs in most xenobiotics. The sulfation process helps in reducing the toxicity of xenobiotics. While the glucuronic acid conjugates are eliminated in bile (those with high molecular weight) and/or through kidney, the highly polar sulfate conjugates are excreted out from the body through urine. In some of the cases, the glucuronidation or the sulfation can conjugate the same xenobiotics. Generally, this sulfation pathway has a low capacity for the xenobiotic conjugation (David et al. 2005; Jancova et al. 2010). For example, sulfate conjugation of phenol to phenyl sulfate (Fig. 16.4).

The common drug which undergoes sulfation is paracetamol. But as described, only 40% of it is metabolized due to sulfation (Schonborn and Gwinnutt 2010).

16.3.2.3 Phase 0 and Phase III Concept or Transporters

The concept of two phase biotransformation reaction given by Professor RT William has been extended to four phases with transporters. Transporters guide the xenobiotics, drugs and their metabolites in entering and excreting out of the cells. In general, the xenobiotics or drugs are not able to overcome the phospholipid membrane barrier. Thus, these transporters help and guide the xenobiotics and drugs to overcome the phospholipid barrier. These transporters belong to two main clusters of transporter families, i.e. solute carrier (SLC) and the ATP binding cassette (ABC) carriers (Döring and Petzinger 2014).

The SLC comprises of 400 different transporters, organized into 65 families and are involved in drug uptake. Later on, this uptake process was termed as "phase 0 transporters or phase 0 reaction" of biotransformation (Fig. 16.5) (Döring and Petzinger 2014; Bai et al. 2017).

Similarly, the ABC transporters have 49 family members belonging to seven subfamilies (Hacker et al. 2009). These carriers operate as pumps at the expense of ATP splitting. In the year 1992, Ishikawa coined the term "phase III reaction" which

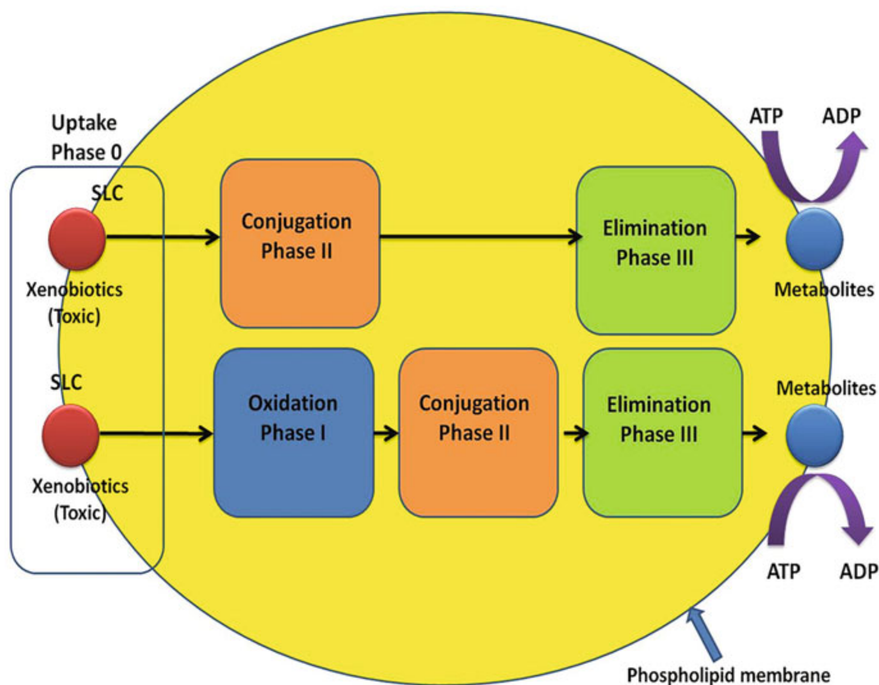

Fig. 16.5 Various steps involved in neutralization and/or elimination of xenobiotics or drugs from the cell inside the living body

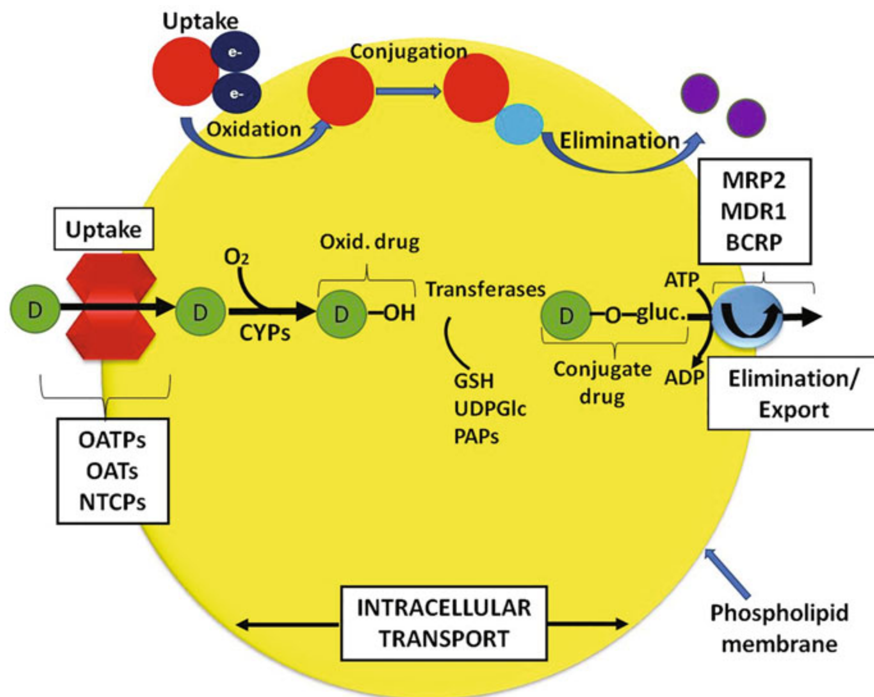

Fig. 16.6 Sequential steps of drug uptake from blood, metabolism inside the cell and its excretion by metabolism and membrane transport pathways via various carriers (Döring and Petzinger 2014; Modified)

includes ABC efflux pumps in the process of biotransformation for the export of drug material (Fig. 16.5).

These transporters for xenobiotics or drugs in eukaryotes generally operate in liver but they are also present in extra-hepatic tissues such as lung, the adrenal gland, intestine, and kidney. As tissues perform specific functions therefore according to their epithelial role (absorption or secretion), the cellular location of the transporters varies. In polarized cell, generally, the SLC transporters are located on blood-facing side of a cell, whereas ABC carriers occur on lumen side or on both sides (Petzinger and Geyer 2006).

The sequential transport steps of a drug molecule in a liver cell are given in Fig. 16.6.

Classification of Transporters

The biotransformation reaction needs transporters for the uptake and elimination process from the cell to extracellular fluid. There are 2 and 16% prokaryotic and eukaryotic genomes, respectively, which encode for the membrane transport proteins (Ren and Paulsen 2005; Hediger et al. 2013). According to current

knowledge, the transporters are divided into two types, i.e. phase 0 transporters and phase III transporters.

Phase 0 Transporters

These are transporters which generally initiate drug metabolism. Phase 0 transporters help in the uptake of drug molecules by using the SLC families. These SLCs operate non-actively as uniporters and act via facilitated biological diffusion, which allows an uptake of extracellular molecules inside the cells. It acts on the theory of concentration gradient (passive transport). The SLC-mediated drug transport might also be secondarily active via symport in the identical flux direction together with an organic substrate or an electrolyte, or in the opposite direction in the case of antiport (Fig. 16.7) (Döring and Petzinger 2014).

Phase III Transporters

Phase III-mediated elimination uses active transport. ABC carriers play an important role in the elimination of metabolites to extracellular fluids. ABC carriers generate a primary active transport process, which means uphill transport as against a concentration gradient of the transported substrate (transportate) using energy by converting

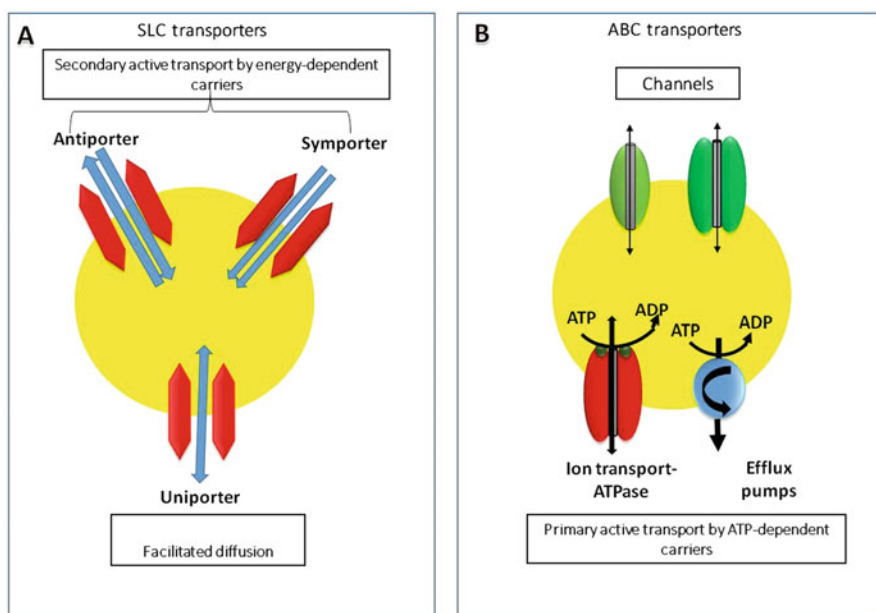

Fig. 16.7 Classification of transmembrane transporters and transport processes for organic compounds and electrolytes. (**a**) SLC carriers are facilitated diffusion carriers that allow non-active downhill transport (uniporter) but, in the case of sym- and antiporters, achieve uphill concentrative substrate transport as well (secondary active transport mechanism). (**b**) The ABC carriers use ATP for uphill substrate transport (primary active transport mechanism) (Döring and Petzinger 2014; Modified)

the ATP to ADP (Fig. 16.7). The ABC carriers including the GS-X pump named multidrug resistance-associated protein 1 (MRP1) (Fig. 16.6) and P-gp (Ishikawa 1992; Hediger et al. 2004).

16.4 Types of Biotransformation

16.4.1 Enzymatic Elimination

The use of different metabolizing enzymes of the body/cells for the process of biotransformation is known as enzymatic elimination. On the basis of compartmentalization of enzymes, it is broadly classified into non-microsomal biotransformation and microsomal biotransformation (Smitha et al. 2017).

16.4.2 Non-microsomal Biotransformation

A type of biotransformation in which the concerned enzymes are soluble and are present in the cytoplasm and mitochondria of cells in tissues including liver, intestine, plasma, etc. (Gaynes and Fiscella 1996). These enzymes catalyze mainly reductive and hydrolytic reactions, a few oxidative, and also conjugation reaction except glucuronidation. This category contains alcohol dehydrogenase, aldehyde dehydrogenase, monoamine oxidase, diamine oxidase, etc. (Gaynes and Fiscella 1996). The examples of reactions catalyzed by these enzymes include:

- Non-microsomal metabolism of catecholamines and noradrenaline via monoamine oxidase.
- Metabolism of ethanol into acetaldehyde by alcohol dehydrogenase.

As these enzymes are present in less quantity or a few are completely absent in neonates, they make neonates sensitive to a few drugs. For example, even though some drugs or syrups contain very less quantity of alcohol, they are still harmful to children as they cannot metabolize the drug completely (Sellers and Holloway 1978).

16.4.3 Microsomal Biotransformation

A type of biotransformation in which enzymes responsible are present within the lipophilic vesicle called microsome (Buhler and Williams 1988). These heterogeneous vesicles are formed from the endoplasmic reticulum when the cells are disrupted in vitro. These range from 20 to 200 nm in diameter and are isolated by differential centrifugation. They possess all functional, morphological properties of the endoplasmic reticulum. These have mainly three structural features: rough vesicles, smooth vesicles, and ribosomes & enzymes associated with it. In general,

the smooth endoplasmic reticulum is concerned with biotransformation and contains enzyme components while the rough endoplasmic reticulum is mainly concerned with protein synthesis (Buhler and Williams 1988).

These are mainly present in tissues like liver, kidney, lungs, and intestinal mucosa. These can be induced by drugs and/or diet. This category contains—Cytochrome P450 (CYPs), Flavin-containing monooxygenase (FMOs), UDP-glucuronosyltransferase (UGTs), etc. (Fig. 16.6) Enzymes of this category are non-soluble proteins. The microsome containing Cytochrome P450 (ferric, ferrous forms), NADPH (flavoprotein), membrane lipids, molecular oxygen is termed as microsomal mixed function oxidase system (Parkinson 2001; Timbrell and Marrs 2009).

The microsomes are very useful for researchers to mimic the properties of the endoplasmic reticulum in a test tube. They use hepatic microsomal preparations from various species for determination of phase I biotransformations of xenobiotics (drugs). The CYP enzyme family present in microsomes is responsible for phase I biotransformations and these tests are the primary source of information on the metabolism of any test compound. For this determination, the test compound is typically incubated in approximately 1.0 mg/ml microsomal protein, phosphate buffer, pH 7.4 at 37 °C for 30 min (Vrbanac and Slauter 2013). Its stability is then checked by an appropriate analytical method such as LC-UV or LC-MS (Vrbanac and Slauter 2013). Viable drug candidates should have a species-specific, pre-determined percentage remaining under these conditions.

16.5 Non-enzymatic Elimination

It is a process of spontaneous, non-catalyzed type of biotransformation which takes place at physiological pH for highly active, unstable compounds (Smitha et al. 2017).

The substrate can react with any of the following to result in a non-enzymatic reaction:

1. Reactions with endogenous nucleophiles: Endogenous water, thiols, cysteine act as endogenous nucleophiles. Example: Oxidation of nitrosobenzene in red blood cells.
2. Reactions with endogenous electrophiles: Biogenic aldehydes and ketones form endogenous electrophiles. Example: Formation of hydrazones from hydralazines.
3. Breakdown and rearrangement in acidic and neutral aqueous media. Example: non-enzymatic conversion reaction of 6-chloromethyl and 6-bromomethyl to 6-hydroxymethyl BP at physiological conditions. 80–90% of the incubated amount was transformed in 1 h.
4. Reactions between themselves (two drug or their metabolites): such reactions usually involve adduct formation. Example co-administration of acetylsalicylic acid and acetaminophen to model animals leads to the formation of salicylic acid

N,O-diacetyl-p-aminophenol. Example 2: At 37 °C and pH 2.5, norethindrone reacts with isoniazid form hydrazone XXI, in 6 min.

Examples Drugs which undergo non-enzymatic elimination:

- Mustin HCl converted into Ethyleneimonium
- Atracurium converted into Laudanosine and Quaternary acid
- Hexamine converted into Formaldehyde
- Clorazepate converted into Desmethyldiazepam

16.6 Factors Affecting Biotransformation

The process of transformation in vitro can be affected by numerous factors (Renton 1986) such as:

1. Physiochemical properties of the drug
2. Chemical properties of the drug
3. Biological factors

16.6.1 Physiochemical Properties of Drug

It includes the molecular size, acidity/basicity, pKa values, lipophilicity, interaction with drug metabolizing enzymes.

16.6.2 Chemical Properties of Drug

16.6.2.1 Enzyme Induction

Certain enzymes get induced or activated when they are exposed to a specific drug. These agents are called enzyme inducers. These can be several drugs and pesticides, or even cigarette smoke.

16.6.2.2 Enzyme Inhibition

Inhibition of enzyme activity due to exposure to a specific drug is a property which affects the biotransformation process. The inhibition can be due to a direct or indirect mechanism. Direct inhibition is further achieved by three mechanisms—competitive inhibition, non-competitive inhibition, product inhibition. Example: allopurinol inhibits xanthine oxidase; whereas indirect inhibition is achieved by two mechanisms, i.e. repression and altered physiology. Examples: actinomycin, puromycin.

16.6.2.3 Environmental Factors/Chemicals

The natural factors which affect the outcome of a biotransformation process can be the presence of halogenated pesticides, polycyclic aromatic hydrocarbons; organophosphate insecticides, and heavy metals in the environment. The temperature, altitude, atmospheric pressure, etc. are other forces which affect the process.

16.6.3 Biological Factors

16.6.3.1 Species Difference

Due to the difference in genetic makeup the drug metabolizing enzymes which are controlled by genes differ from species to species.

Example Dogs are deficient in acetyltransferase and cats are deficient in glucuronosyltransferase.

16.6.3.2 Strain Difference

The difference observed in response to the same drug within the individuals of the same species is called pharmacogenetics. The strain difference is also termed as the study of inter-subject variability in drug response. It can be monogenetically or polygenetically controlled. In humans, polygenic control is observed in fraternal twins, born from different eggs. But variations also arise due to ethnic cultural differences.

16.6.3.3 Sex Difference

When the regulation of drug metabolism is controlled by sex hormones, it causes variation in the process in different sexes of the studied population.

16.6.3.4 Age

As the body's growth status is at a different page in different age groups, the process of biotransformation varies among children, adults, and elderly. As mentioned before, the neonates and infants have underdeveloped microsomal enzyme system so they cannot metabolize commonly used drugs containing alcohol. But on the other hand, children can metabolize some drugs faster than adults. Whereas in elderly the reduced liver size and decreased hepatic blood flow also affect the biotransformation process.

16.6.3.5 Diet

The type of food intake forms a very important factor which affects the enzyme system in the body thereby affecting the whole process. The total protein intake; protein–carbohydrate ratio; fat-free diet; dietary deficiency of vitamins and minerals; starvation; alcohol ingestion are important parameters to consider for drug metabolism.

16.6.3.6 Altered Physiological Factors

The physiological conditions like being pregnant, diseased, or suffering from hormonal imbalances have drastic effects on the biotransformation process of several drugs. These states can reduce the individual's drug metabolizing ability due to high steroid hormones, or enhance half-life of almost all the drugs or may inhibit the activity of few enzymes while may induce others.

16.7 Biological Agents Used for Biotransformation

Biotransformation process has been witnessed by humans from the ancient times but was not understood then. The products of transformations have been extensively used and enjoyed for thousands of years for manufacturing bread, dairy products, and alcoholic beverages. Ethanol to vinegar was probably the first true biotransformation process applied in an industrial manner.

In a broad sense, biotransformation refers to the process in which microorganisms convert organic compounds into structurally related products. Microbes develop mechanisms to acclimatize to changing environmental conditions, thus proving very useful as biological agents or biocatalysts for biotransformation processes.

Certain reasons have been listed by researchers which make microbial cells as an ideal choice for biotransformation over conventional chemical methods (Smitha et al. 2017) such as:

1. *Surface–volume ratio*: Microbial biotransformation needs a high surface–volume ratio in the microbial cells.
2. *High growth rate*: The higher growth rate of microbial cells reduces the time of biomass transformation.
3. *High metabolic rate*: The higher rate of the metabolism in microbes leads to efficient transformation of the substrate.
4. *Toxic waste products*: Microbial biotransformation usually produces reduced levels of toxic waste products.
5. *Non-extreme conditions*: A highly selective biotransformation procedure can be performed with microbes at non-extreme pH and near room temperature.
6. *Desired stereoselectivity*: Use of specific microbes for regio- and stereoselective reactions, or to introduce chirality in end products.

These biocatalysts or the agents are not limited to microbes as whole cells. In fact, the enzymes are the principal biocatalysts. Their properties were recognized in the early 1900s during the phase of kinetic studies (Lin and Tao 2017).

16.7.1 Enzymes

These are key agents of biotransformation process. They are known to catalyze innumerable different chemical reactions. Therefore, these are named and classified into six classes accordingly by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) (McDonald et al. 2001).

1. *Oxidoreductases*: Enzymes catalyzing oxidation-reduction reaction, i.e. oxygenation of C–H, C–C, C=C bonds, or overall removal or addition of hydrogen atom equivalents belong to this category.
2. *Transferases*: Enzymes catalyzing the transfer of a group from one compound to another belong to this category. The different groups can be a methyl, aldehydic, ketonic, acyl, sugar, or phosphoryl.
3. *Hydrolases*: The enzymes which catalyze hydrolytic cleavage of C–O, C–N, C–C bonds. Hydrolases catalyze the removal of groups from their substrate to acceptor water molecules.
4. *Lyases*: These enzymes catalyze the addition or elimination of small molecules on C=C, C=N, C=O bonds.
5. *Isomerases*: These enzymes catalyze structural changes like racemization, epimerization, a rearrangement in a molecule.
6. *Ligases*: Ligases are enzymes catalyzing the joining together of two molecules coupled with the hydrolysis of a diphosphate bond in ATP or a similar triphosphate.

All the above-mentioned enzymes participate in the defined phases of biotransformation. The physical state of these biological agents whether isolated enzyme systems or intact whole cells varies depending on the following factors (Doelle et al. 2009):

1. The type of reaction.
2. The requirement of co-factors.
3. The scale at which the biotransformation has to be performed.

16.7.2 Immobilized Enzymes

The enzymes restricted to a polymeric matrices or a carrier material by a physical or chemical treatment form an immobilized enzyme system. Although there is eventual loss of activity during immobilization of enzymes, these cell-free enzyme systems are commonly used in biotransformations, due to the following advantages:

1. The single desired product is mostly the end product as undesirable side reactions do not occur.
2. The desired products are stable and do not degrade.

3. As it is a cell-free system, there is no transport barrier such as cell membrane for the substrate or product.
4. The isolation and recovery of the product are simpler and easier.
5. Not only the product but the system itself is stable.

Example Production of glucose isomerase, penicillin acylase.

16.7.3 Whole Cells

Isolated immobilized enzyme systems are easily available commercially and have advantages of their own. However, the need of a cofactor for the specific reaction adds complications to the process. The whole cell systems have complete machinery for enzymes thus they overcome the disadvantage of co-factors. This system is also environmental friendly and cheaper than isolated enzyme systems. However, the major disadvantages of the system are then tiresome work-up due to large volumes, low productivity due to lower concentration tolerance, the formation of more than one product due to side reactions, and cell membrane as a barrier for biomolecule transportation.

Example The production of aromatic alcohol 2-phenylethanol (2-PE) from l-phenylalanine (l-Phe) in yeast. The use of whole cells for production of the suitable product can be performed in the form of growing cells or non-growing cells.

16.7.4 Growing Cells

Growing whole cell system is the most common type of production systems. Here the desired cells are cultivated in a suitable medium. A concentrated substrate is added to the culture only after a certain growth of the cells in the media. Sometimes, emulsifiers (Tween, organic solvents) are added to solubilize substrates and/or products as in case of steroid biotransformation. The substrate conversion to the product can be monitored by spectroscopic or chromatographic techniques. Biotransformation can be terminated when the product formation is optimum.

16.7.5 Non-growing Cells

During the non-growing cell biotransformation process, the cell growth (the enzyme manufacturing phase) and production phase are separated. Substrates are converted to the desired products by resting or non-growing cells. It is a preferred method for biotransformation reactions due to the following advantages:

1. A very high substrate concentration can be used as the cell growth stops at this concentration.

2. Low chances of contamination as cells can be washed and used again.
3. The conversion efficiency of the substrate to product is high.
4. Product isolation and its recovery are easy.

Example Free resting cells of *Mycobacterium* sp. NRRL B-3805 used for side-chain cleavage of β-sitosterol in biphasic systems containing bis(2-ethylhexyl) phthalate (BEHP).

16.7.6 Immobilized Cells

Biotransformations can also be carried out using the immobilized living cells with a specific set of enzymes. The same cells can be used again and again. The transformation mechanism can be a single or multistage reaction.

Example Commercial production of malic acid and L-alanine. The use of lipases by immobilization of fungal mycelium on biomass supports particles and the expression of lipases on the surface of microbial cells is another example.

16.8 Product Recovery in Biotransformation

In commercial times, the biotransformation is employed to yield a specific product, and in most of the cases, the end product is extracellular. It can be in the soluble state or in a suspended state. Accordingly, the product is recovered by using common techniques like extraction using solvents, precipitation with salts, adsorption, and ion exchange chromatography method. The volatile products can be recovered by direct distillation from the medium (Hellstén 2013; Fan et al. 2014).

16.9 Advantages of Biotransformation

- This technique is helpful in the production of those compounds which are otherwise difficult to be produced via chemical methods (Sultana and Saify 2013).
- Biocatalysts are highly substrate specific, stereo specific. They can easily incorporate desired structural changes than chemical reactions (Sultana and Saify 2013).
- Recombinant DNA technology can be easily applied to microbes for production of desired end product in biotransformation (Vasic-Racki 2006).
- Further, biotransformation with recombinant microbial enzymes has been widely used, including applications for the production of hormones, antibiotics, and specialty chemicals (Seeger et al. 2003).

16.10 Current Status of Biotransformation Technique

Scientists all around the world are exploring the biotransformation process for drug designing and development. The success of a safe drug designing programme lies in proper knowledge and combination of pharmacoproperties of molecule/s under consideration. Thus the drug metabolism research plays the most important role in its designing programme (Kebamo et al. 2015). Therefore, a lot of interest in the industrial research lies on the biotransformation of bioactive molecules in the body. Indeed very recently, the first commercial software was introduced in the market for automated catabolite and metabolite identification by SCIEX company which offers the flexibility to quickly identify metabolites, catabolites and enable in-depth metabolism or catabolism studies (Sicurella and Farrell 2017). The biotransformation information not only helps in designing and development but also in the production of several drugs such as steroid based pharmaceutical products, anticancer drugs, regio-, stereo-specific drugs from natural raw materials (Gao et al. 2013; Song 2018). The knowledge of this process also enables and strengthens the researchers to use microbes for production of bioactive metabolites, antifungal products, etc. under the concepts of white biotechnology and green chemistry (Bianchini et al. 2015). Also, with the help of metabolomics approach, researchers are trying to study the biotransformation biomarkers of metabolomic pathways related to certain diseases to get an insight for development of the disease.

Development and globalization in the countries around the world have produced various side effects on the environment in the form of pollutants. Various conventions have been signed and the governments are funding researches to build strategies to employ the biological processes for pollution abatement. The countries are guiding their industries to adopt and integrate methods to treat their effluents by natural process with environmental, economic, and social acceptability. Thus, biotransformation of environment pollutants like petroleum hydrocarbons, dye-contaminated industrial effluents, heavy metals like mercury and chromium, municipal waste, aquaculture effluents is looked up as vital solution for environment crisis. Various technologies have been patented which use the microbes like pseudomonas spp., thermus spp., *Archaeoglobus fulgidus*, *Microbacterium* sp., etc. in consortia for cleaning the environment (Peeples 2014). Researchers are thus continuing to evaluate this process as solution for providing more specific drugs, for disease development, reducing pollution, etc. and it is anticipated that biotransformation will be part of sustainable solution to a better future.

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