Pradeep Verma *Editor*

Industrial Microbiology and Biotechnology

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ISBN 978-981-16-5213-4 ISBN 978-981-16-5214-1 (eBook)
https://doi.org/10.1007/978-981-16-5214-1

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Dedicated to My Beloved Mother

Preface

Microorganisms are ubiquitous and are a rich source of bioactive compounds and have been explored for ages for human application. Humans have learned ways to use microorganisms from the preparation of curd, cheese, wine, alcohol, etc. The production of insulin and penicillium can be considered as the arrival of modern industrial microbiology. Several biotechnological approaches have been developed to harness the potential of microbes. It has been considered as a separate stream of study, often referred to as microbial biotechnology. These approaches utilize microorganisms for the production of industrially important products through greener approaches rather than traditional chemical and energy-intensive processes. As a result, the shift has occurred towards microbes-based greener approaches for industrial application. The advent of modern biotechnological techniques has led to considerable research and progress in the area of industrial microbiology. Microbes-based products have a widespread application from agriculture, food processing, paper, and pulp industries to biorefinery, bioremediation, pharmaceutical, and medical sectors. Therefore, there is an urgent need to understand the progress made in the field of industrial microbiology and biotechnology intended to have large-scale modern industrial applications.

The present book is an attempt to provide its readers with compiled and updated information in the area of Industrial Microbiology and Biotechnology. This book provides the basics of microbiology and how it has been exploited at an industrial scale. The book focuses on the role of biotechnological advances that directly impact the industrial production of several bioactive compounds using microbes-based methods under a controlled and regulated environment. On the one hand, this book presents detailed information on the basics of microbiology such as types of microbes and their applications, bioreactor design, fermentation techniques, and strain improvement strategies. On the other hand, it also provides recent and updated information on industrial production, recovery, and applications of enzymes, alcohols, organic acids, steroids as a drug precursor, etc., using microbial biotechnological approaches. The book presents an overview of modern technological advances for the generation of energy (biomethane, bioethanol, and bioelectricity) and resource recovery from waste. It also highlights the application of CRISPR-based technologies in the industrial microbiology sector. This book is developed with the motive to benefit students, academicians, as well as researchers. The book will also be of interest to microbiologists, biotechnologists, environmentalists, and

engineers working in the application of the microbes-based approach for the development of greener technologies.

Ajmer, Rajasthan, India Pradeep Verma

Acknowledgments

First of all, I would like to convey my gratitude to Springer Nature for accepting my proposal to act as editor for the current book volume. The current volume of the book series could not be possible without the support of all the researchers and academicians who contributed to the book. Their quick response to my invitations and during the revision stage supported immensely; therefore I am thankful for their contributions. I would also like to thank my Ph.D. scholar, Mr. Bikash Kumar, for providing me with all the necessary support during review/editing process, compilation of chapters, and technical support during the entire stage of book development. I am also thankful to the Central University of Rajasthan (CURAJ), Ajmer, India, for providing infrastructural support and a suitable teaching and research environment. The experience of teaching graduate, postgraduate, and young researchers at CURAJ provided the necessary understanding of the needs of academicians, students, and researchers in an industrial microbiology book that was greatly helpful during the development of the book, and also made all possible attempts to overcome existing limitations in the literature. I am also thankful to the Department of Biotechnology for providing me funds through sponsored projects (Grant No. BT/304/NE/TBP/2012 and BT/PR7333/PBD/26/373/2012) for setting up of my laboratory, "Bioprocess and Bioenergy Laboratory."

I am always thankful to God and my parents for their blessings. This book is affectionately dedicated to the most caring and loving women (mother and wife) in my life. Their love, care, and trust gave me strength and motivation to contribute in the scientific world. I also express my deep sense of gratitude to my wife and kids for their support during the development of the book and in life.

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Editor and Contributors

About the Editor

Pradeep Verma completed his PhD from Sardar Patel University, Gujarat, India, in 2002. In the same year, he was selected as a UNESCO fellow and joined the Czech Academy of Sciences, Prague, Czech Republic. He later moved to Charles University, Prague, to work as Post-Doctoral Fellow. In 2004 he joined as a visiting scientist at UFZ Centre for Environmental Research, Halle, Germany. He was awarded a DFG fellowship which provided him another opportunity to work as a Post-Doctoral Fellow at Gottingen University, Germany. He moved to India in 2007 where he joined Reliance Life Sciences, Mumbai, and worked extensively on biobutanol production, which attributed few patents to his name. Later he was awarded JSPS Post-Doctoral Fellowship Programme and joined the Laboratory of Biomass Conversion, Research Institute of Sustainable Humanosphere (RISH), Kyoto University, Japan. He is also a recipient of various prestigious awards such as the Ron-Cockcroft award by Swedish Society and UNESCO Fellow, ASCR, Prague.

Prof. Verma began his independent academic career in 2009 as a Reader and Founder Head in the Department of Microbiology at Assam University. In 2011 he moved to the Department of Biotechnology at Guru Ghasidas Vishwavidyalaya (A Central University), Bilaspur, and served as an Associate Professor till 2013. He is currently working as a Professor (former Head and Dean, School of Life Sciences) in the Department of Microbiology, CURAJ. He is a member of various national and international societies/academies.

He has completed two collaborated projects worth 150 million INR in the area of microbial diversity and bioenergy.

Prof. Verma is a Group leader of the Bioprocess and Bioenergy Laboratory in the Department of Microbiology at the School of Life Sciences, CURAJ. His area of expertise involves Microbial Diversity, Bioremediation, Bioprocess Development, Lignocellulosic, and Algal Biomass-based Biorefinery. He holds 12 international patents in the field of microwave-assisted biomass pretreatment and biobutanol production. He has more than 60 research articles in peer-reviewed international journals and contributed to several book chapters (28 published; 15 in press) in different edited books. He has edited three books for international publishers. He is an editorial board member, guest editor and reviewer to several high impact peer-reviewed international journals.

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Understanding the Small World: The Microbes

1

Pompee Chanda and S. R. Joshi

Abstract

One cannot see the microbes which, in fact, are the leading entity of life on Earth. Other than their widespread occurrence in natural habitats such as the soil and the aquatic environments, microbes also thrive in extreme environments like arctic waters and hot springs. They add up to half of the world's biomass. The natural habitats are the source of microbes. They inhabited these habitats billions of years ago even before the humans came to their existence and are expected to remain and dominate the Earth even after humans are gone. Without their presence, life, as we know it, would not have been possible. Major part of our body mass contains more bacterial cells as compared to cells in the human body. Undeniably, the relationship between microbes and humans is delicate and intricate. Understanding the small world—the microbes—provides a clear and accessible introduction on how microbes impact our lives and are involved both in good health and sickness through their benefit and harm. This chapter provides content on how microbes are isolated and cultured, and strain improvement techniques are undertaken to extract useful or novel products which have extensive applications in biotechnology industry. This interactive and engaging chapter provides the basics of microbiology, in a contemporary context. It will be equally useful in the fields across the biological, environmental and health sciences, and for the interested reader wanting to explore more about these ubiquitous microbes.

Keywords

Microbes · Habitats · Ubiquitous · Distribution · Applications

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_1

Abbreviations

70S	70 Svedberg units
80S	80 Svedberg units
AFM	Atomic force microscope
AIDS	Acquired immunodeficiency syndrome
ASBVd	*Avocado sunblotch viroid*
BSE	Bovine spongiform encephalopathy
CCCVd	*Coconut cadang-cadang viroid*
CEVd	*Citrus exocortis viroid*
cfu	Colony-forming units
CJD	Creutzfeldt-Jakob disease
COVID	Coronavirus disease
CPrP	Cellular prion protein
CSVd	*Chrysanthemum stunt viroid*
DICM	Differential interference contrast microscopy
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EM	Electron microscope
GI	Gastrointestinal
GPI	Glycosyl phosphatidyl inositol
HDV	Hepatitis delta virus
HMP	Human Microbiome Project
HSV-1	Herpes simplex virus type 1
ICTV	International Committee on Taxonomy of Viruses
IR	Infrared
mBRC	Microbial biological resource centre
MERS	Middle East respiratory syndrome
MPN	Most probable number
mRNA	Messenger ribonucleic acid
NMR	Nuclear magnetic resonance
PAMP	Pathogen-associated molecular pattern
PSTV	Potato spindle tuber viroid
RNA	Ribonucleic acid
SARS	Severe acute respiratory syndrome
SEM	Scanning electron microscopy
SPM	Scanning probe microscopy
STM	Scanning tunnelling microscope
TEM	Transmission electron microscopy
TMAO	Trimethylamine N-oxide
TMD	Tobacco mosaic disease
TPMVd	Tomato planta macho viroid

1.1 Introduction to Microbiology and Microbes

Microbiology is the study of the structure, activity, and categorization of bacteria with the goal of manipulating and regulating their behaviour (Gross et al. 1995). It all began with Antonie van Leeuwenhoek's pioneering study on microbes in the seventeenth century, which he conducted with a self-designed microscope (Gest 2004). A microorganism can be categorized into two groups—one that contains cells termed as cellular and the other without cells termed as acellular. The first category of microbes comprises bacteria, archaea, fungi and protists. The protists belong to the kingdom Protista. Algae, protozoa, slime moulds, and water moulds are among the microbes that belong to the kingdom. This type of microbes can be either single celled (unicellular) comprising the complete organism, or they can be aggregates of many number of cells which usually range from hundreds to billions of cells (multicellular), which constitute the entire organism. Microbes such as viruses, prions, and viroids, are found in the acellular category and are considered infectious agents (Pepper et al. 2014). Our everyday existence is intertwined inseparably with microbes. Microbes not only inhabit the human body; they are also found to thrive in soil, in hot springs, in deep oceans, in high altitude atmosphere and also deep in rock bed (Orcutt et al. 2011). Microbes are invisible, yet seem to be abundant on Earth; they provide sufficient evidence of its existence in both negative and positive ways. They can cause spoilage when they cause decomposition and diseases. And at the same time of various advantages to mankind., which include its application in various industries such as fermentation, food, pharmaceuticals, etc. (Sharma et al. 2020). Most importantly, microbes are vital in maintaining the ecological balance of the Earth. They decompose animal and plant remnants and convert them to biodegradable and recyclable substances. Thus, they have an incalculable contribution to the Earth's biogeochemical cycle—the carbon cycle and the nitrogen cycle (Rousk and Bengtson 2014).

1.1.1 Microbes

The term *microbe* was used for the first time in the late nineteenth century to describe all the invisible life forms existing on Earth. Professor Charles Sédillot (1804–1883) the pioneer of modern medicine, surgery, anaesthesiology, histopathology and infectiology was the one to first use the term microbe. Long before the explanation on infections was put forth by Semmelweis (1818–1865), Professor Charles Sédillot predicted the existence of microbes and understood their action mechanism, which he found to develop as postoperative infections (Billmann 2012). As microbiology in due course of time developed into a specialized field of study in biology, it was discovered that microbes are congregation of varied organisms which are microscopic, suggesting the use of an external support, the microscope, in order to see them. Of course there are exceptions. Some unicellular organisms studied by microbiologists are macroscopic. These include *Valonia ventricosa* (Guiry and Guiry 2021), for example, can grow up to 5 cm in length. They're microscopic

algae that go by the names "bubble algae" and "sea pearl." Each bubble is made up of a single cell and is considered one of the world's largest single-celled organisms. They are unique sorts of algae species that are widely distributed and have the ability to survive low light. They live in mangrove forests and are considered pests because they grow on their roots. *Thiomargarita namibiensis*, another variety of macroscopic organism with diameters ranging from 100 to 750 micrometer (Schulz and de Beer 2002), was also discovered.

1.1.2 The History of Uncovering the Mystery of Microorganisms

The existence of microbes was first reported during the late sixteenth century by two most distinguished fellows of the Royal Society, Robert Hooke and Antonie van Leeuwenhoek. Antonie van Leeuwenhoek, a cloth draper by profession was the first to watch microbes, with the aid of his self-designed microscope, and thus made one of the most remarkable contributions in the field of biological sciences. Seemingly, he found pleasure in screening of microbes in samples from different sources such as water from the pond, faecal wastes, scrapings from the teeth, etc. Antonie van Leeuwenhoek made comprehensive diagrammatic illustrations to document his findings in detail. Which was later conveyed to the scientific society of that time the Royal Society of London. Robert Hooke published a book *Micrographia* in the year 1665. In this book for the first time, Robert Hooke formally introduced his published illustration of a microorganism, which is a microfungus termed as *Mucor*. Such breakthrough discoveries led to the conception and using of simple microscopes that could magnify objects from about 25- to 250-fold. As a result, about a century and a half after its discovery, the microscope has become the most significant aspect of human consciousness for comprehending the role that bacteria played in generating infections in many life forms and organisms (Gest 2004). Other eminent scientists also made their contributions to microbiology.

- Lazzaro Spallanzani, an Italian physiologist and biologist, was not far behind in contributing towards the investigation of microbes. His detailed research on biogenesis debunked the hypothesis of spontaneous generation. He discovered that boiling the broth sterilised it and killed any germs contained in it, but that leaving it out in the open may lead to infection (Mancini et al. 2007). His findings paved the way for the future research of Louis Pasteur who had ultimately put a dead end to the doctrine of biogenesis and supported germ theory instead (Sharma et al. 2020). Thus, with the discoveries of Van Leeuwenhoek, Lazzaro Spallanzani, and others, the hypothesised notion that life arose spontaneously from non-living objects during the decay process was disproved.
- Ferdinand Julius Cohn, a biologist from Germany and a pioneer in developmental biology of lower plants, was the first to categorize algae as plants and to give explanation as to what property discriminates algae from green plants. He significantly encouraged the taxonomical and physiological classification of bacteria. He divided the bacteria into four different groups on the basis of shapes, namely,

spherical (coccus), rod-shaped (bacillus), comma-shaped (vibrio) and spiral (spirilla). This type of bacterial nomenclature is being used till date. Also Cohn has proposed that Bacillus has the ability to change its state from vegetative form to an endospore when subjected to an unfavourable environment (Drews 2000).

- A Prussian physician and microbiologist Heinrich Hermann Robert Koch, the pioneer of contemporary bacterial study, was the first one to identify the infectious agent behind the diseases like tuberculosis, cholera and anthrax which killed humans as well as animals in the past. He also provided the experimental evidence for the same. He later established Koch's postulates, which consist of a sequence of four universal principles that states specific organisms are causing specific diseases (Segre 2013). Koch was the recipient of the Nobel Prize in Physiology or Medicine in the year 1905. The Robert Koch Institute is named to honour his contribution in the study of bacteriology.

1.1.3 Types of Microorganisms

Microbiology came into being with the studies and investigations of bacteria. As already stated the research of Louis Pasteur and other scientists in the late eighteenth century helped in establishing the fact on how important microbes are in our lives. It was due to their unwavering effort which led to the invention of new techniques for the thorough examination of bacteria using microscopes. However, the technique was later modified to study other microbes other than bacteria, therefore the expansion of bacteriology to microbiology. Microorganisms can exist as unicellular, multicellular or cell constellations. They can be divided into six major types: bacteria, archaea, fungi, protozoa, algae, slime mould and viruses.

1.1.4 The Two Types of Cells: The Eukaryotes and Prokaryotes

Microscopic examination of microbial cell has affirmed the existence of two types of cellular structure: larger cells having complex interior and smaller cells with simple internal organization. Larger cells are known as eukaryotes and the smaller cells are known as prokaryotes. At the ultrastructure level, eukaryotes have an internal structure composed of membranes that divide the cytoplasm into a number of different compartments such as nucleus containing DNA molecule wrapped up in filamentous or thread-like structures termed as chromosomes, whereas prokaryotes lack proper membrane-bound nucleus. The DNA of the prokaryotes is scattered as a long, folded strand randomly within the cell. Despite being small and simple, prokaryotes are considered to be highly organized structure, though the organization is at molecular level and not easily visible through the naked eyes. Prokaryotes grow rapidly by utilizing the nutrients dissolved in the liquid surrounding them. Thus prokaryotes have evolved with a permeable structure, highly efficient and having tightly regulated metabolic system to support their survival. On the other hand eukaryotes depend on other species for their survival. They are predators. Example

of such eukaryote are protozoans. Protozoans are free-living single-celled eukaryotes. They live by feeding on smaller prokaryotes for which they occupy the higher hierarchical level in the ecosystem. As such their size has increased and has become more complex in their structure, and over the years they have evolved into multicellular species like algae, fungi and plants (Wheelis 2008). Together algae, protozoa and some lower fungi are grouped as protists (Corliss 1984).

1.1.5 The Three Lineages of Microbial Life

Life forms on Earth are thought to have developed from a common ancestor roughly 3.8 billion years ago. It is thought that the first organism to exist was prokaryotic in nature, with a much simpler structure, followed by the current prokaryotes, which endured hard settings such as hot springs and oxygen deficiency, and had to split into three lineages to adapt further to such circumstances. Accordingly, they were divided into three groups: eubacteria, archaebacteria, and eukaryotes. Approximately around 20 sublineages of plant and animals have been added as new members of the microbial family (Wheelis 2008, Fig. 1.1).

1.1.6 The Prokaryotes (Bacteria)

Bacteria or eubacteria or "true" bacteria form the kingdom (Woese and Fox 1977). They belong to the kingdom Monera. All members of the Monera kingdom are prokaryotes without any nucleus or organelles. Bacteria are unicellular organisms, present everywhere in the biosphere (air, soil, water). They are found in extreme variety of habitats such as boiling thermal springs or in ponds which are either acidic

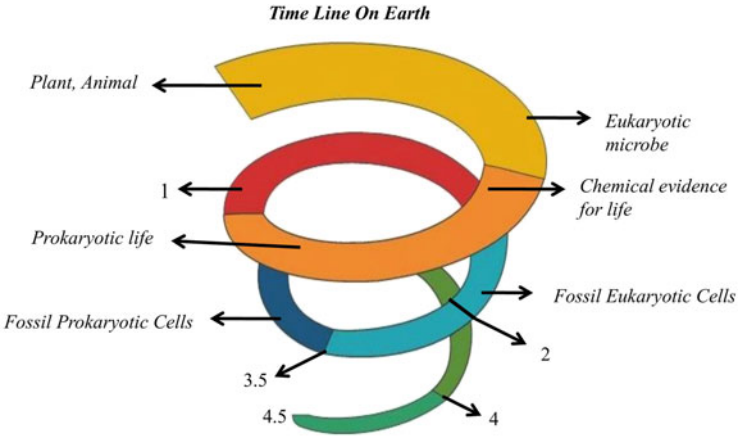

Fig. 1.1 Timeline of life on Earth. The numbers in the figure depict years in billion. (Adopted and modified from Wheelis 2008)

or alkaline. Bacteria have specialized cellular structure. Each bacteria is encased in a rigid cell wall formed of peptidoglycan, a protein-sugar (polysaccharide) polymer. The cell wall protects the cytoplasmic membrane from the environment and provides the cell its form. The cytoplasm, or protoplasm, of bacterial cells is where cell growth, metabolism, and replication take place. It's a gel-like matrix that contains ribosomes, chromosomes, and plasmids (it is distinct from the bacterial chromosome and capable of replication independently). The cell envelope encases the cytoplasm and all of its constituents. Although it is not contained in the nucleoid, the chromosome, which is a single continuous strand of DNA, is positioned there. The cytoplasm is populated with the rest of the cellular components. Some bacteria may have a capsule which is an additional layer that gives protection and have flagella for movement. Bacterial cells can exist in different shapes. They are divided into five types based on their basic shapes: spherical (cocci), rod (bacilli), spiral (spirilla), comma (vibrios), and corkscrew (corkscrew bacteria) (spirochaetes) (Fig. 1.2). Bacteria have many special characteristics. Bacteria have unique way of reproducing. They multiply by the process of binary fission. Bacteria have properties that classify them into different categories. One such feature is the structure of cell wall which classifies them into two groups—gram-positive bacteria and gram-negative bacteria. Gram-positive bacteria have a thick layer of peptidoglycan and lack outer lipid membrane, while gram-negative bacteria have a thin peptidoglycan layer surrounded by lipid membrane. Based on this property, the bacterial cells decide how they take up the stain, hence the names gram-positive bacteria and gram-negative bacteria. Bacteria can be further classified based on how they respond to the

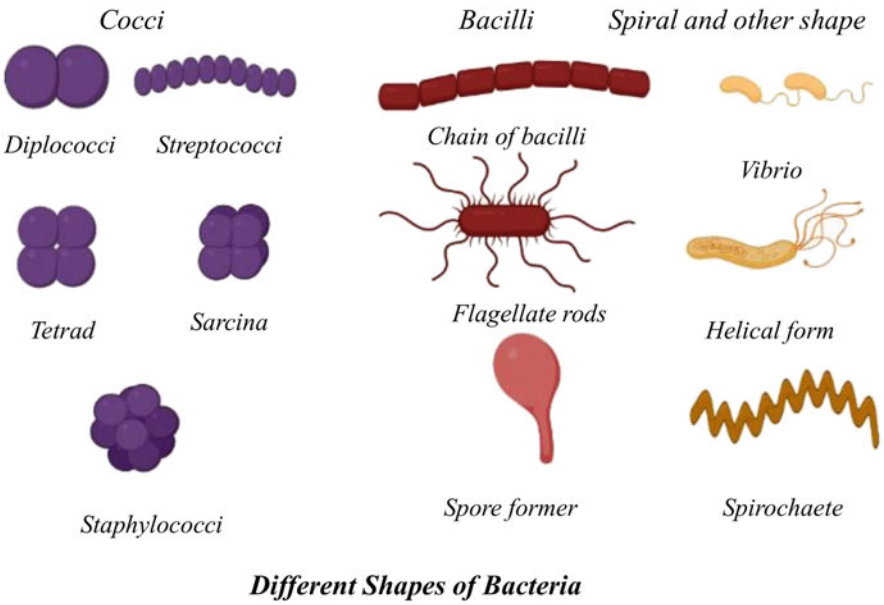

Fig. 1.2 Different shapes of bacteria

presence or absence of atmospheric oxygen into the following groups: They can be aerobic surviving with the available oxygen, anaerobic surviving without oxygen and the one which can live both in presence and absence of oxygen called facultative anaerobe. Bacteria can be further divided on their mode of nutrition. They can be classified as heterotrophs or autotrophs. Autotrophs are able to make their own food by either utilizing the energy from the sun termed as photosynthetic bacteria or using chemical energy, in which case they are called chemosynthetic bacteria. Furthermore according to their habitat, source of food and their association with other organisms, heterotrophic bacteria can be saprophytic which feed on decaying organic matter or parasitic, living on other organisms. They may further be categorized according to the type of environment they live in. Hyperthermophiles grow optimally between temperatures above 80 °C and higher (Uehara et al. 2017), and microbes that can survive extremes of pH are grouped as either acidophiles, which grow optimally below pH 3, or alkaliphiles, organisms that grow at an optimum pH between 8 and 10 (Tiquia-Arashiro and Rodrigues 2016).

1.1.7 The Prokaryote (Archaea)

Archaea form the considerable part of the microbial biomass on Earth. Around the late 1970s, it was reported, on the basis of rRNA phylogenetic studies, that archaea (archaebacteria) were recognized as a separate domain of biological life in addition to bacteria (eubacteria) and eukaryotes. Archaea exhibit a huge variety of lifestyles and metabolic activities. Many archaeal species defy all odds to tolerate the harshest condition from extreme salinity to high temperatures such as hot water vents, hot springs of the land and extreme saline, acidic and anaerobic situations (Schäfer et al. 1999). For example, *Pyrolobus fumarii can stay alive in* hydrothermal vents *at* a maximum of 113 °C (235 °F) temperature. *Picrophilus*, an acidophilic species, is found in the acidic hot springs and dry hot soil in Hokkaido (Japan) capable of survival around pH of −0.06 (Schulze-Makuch and Irwin 2008). Methanogens are anaerobic microorganisms that create methane as a metabolic byproduct. They live in wetlands, where they create marsh gas, and marine sediments, where they make methane (Kristjansson et al. 1982), as well as other severe environments such as hot springs, subsurface hydrothermal vents, and the Earth's crust. They are also responsible for the presence of methane in the digestive tracts of ruminant animals and humans, causing ruminant burping and human flatulence (Lengeler et al. 1999). Though archaea have some characteristics in common with bacteria, they also have several characteristics that set them apart (Niederberger 2020). In archaea, unique ion-transduction processes, the presence of protein complexes in membranes, and the application of novel cofactors in bioenergetics of methanogenesis have all been discovered (Schäfer et al. 1999). Archaea are critical to maintaining the Earth's geochemical cycles, such as the carbon and nitrogen cycles (Offre et al. 2013). Some archaea, such as Thaumarchaeota, are engaged in the nitrogen cycle's conversion of ammonia to nitrite (Kimble et al. 2018). They actively contribute to greenhouse gas emissions (Welte 2018).

1.1.8 The Eukaryotes (Algae)

Algae constitutes a consortium of different primordial plants which comprise diverse evolutionary lineages and are photoautotrophic in nature. All the different groups are of polyphyletic origin and represent the majority types of existing plants. Algae are thallophytes. Their vegetative body unlike plants does not differentiate into roots, stems or leaves. They are non-vascular, photosynthetic and chlorophyll-bearing organisms. Many algae live in lonely cells, primordial plant bodies. Algae are cryptogams that proliferate by using secret reproduction plan. They are mostly inhabitants of humid and aquatic environment (Likens 2009). Through the evolution, algae have formed a number of symbiotic relationship with different types of organisms ranging from other microbes to animals and have integrated their chloroplasts into other eukaryotes. Green algae show many complexity in their organization; they can be both unicellular and multicellular thalli having the length around 120 m. Algae also comes in different shapes. Algae which are single-celled can be spherical, rod-shaped, club-shaped or spindle-shaped. Some algae are motile in nature. They have wide variety of applications. Commercially, they are employed as feed as well as in the food and pharmaceutical industries (Lewis et al. 2011; Pelczar 2020).

1.1.9 The Eukaryotes (Fungi)

Fungi are a branched mass of tubular filaments surrounded by a solid cell wall. The thallus, or undifferentiated section of a typical fungus, is made up of hyphae, which are filament-like structures that create a branched and repeating network termed the mycelium. Organelles and nuclei of the fungi are membrane-bound. Because fungus lacks chlorophyll, it is unable to perform photosynthesis. Fungi, like animals, are heterotrophs, meaning they get their nourishment by absorbing dissolved chemicals and secreting digestive enzymes into their surroundings. The growth of the mycelium depends on the nutrients from the surrounding environment, and at maturity they form reproductive cells called spores. Fungi are eukaryotic organisms that include microbes like yeast, rusts, smuts, mildews and moulds, as well as the mushrooms. There are certain species which are fungi like. They are slime moulds and oomycetes (water moulds). Fungi are both microscopic and macroscopic in size. Macroscopic fungi include mushrooms and bracket fungi that are found to grow in places which are damp. Multicellular fungi include filamentous structure such as moulds, and unicellular fungi include yeasts. Moulds have cylindrical cells that bind from one end to the other to form a thread-like filamentous structure called hyphae which again intricately intertwine to form mycelia. Yeasts on the other hand may be spherical or oval-shaped to filamentous. Though fungi are free-living terrestrial and aquatic bodies, they still can be in symbiotic relationships with other flora and fauna. The mode of nutrition of fungi is heterotrophic as mentioned above. When fungi feed upon a living plant or animal, they are known as biotrophs, and when they obtain their nutrients from dead plants or animals, they are known as saprotrophs

(saprophytes, saprobes). They help in the breakdown of organic compound to release carbon, oxygen, nitrogen and phosphorus into the atmosphere. When a fungi feed on a host by killing, it is known as necrotrophs. Fungi have many important commercial applications also (Hyde et al. 2019). Fungi are used in industry to raise bread dough and produce wine, beer and cheese by fermentation. Some fungal species such as mushroom and Fusarium *venenatum* are used as food grade protein source (González et al. 2020). The latter produces a protein known as mycoprotein (Gilani and Lee 2003). Also fungi are not far behind in contributing to medicine. The discovery of penicillin by Alexander Fleming was a breakthrough in the field of medical history (Tan and Tatsumura 2015).

1.1.10 The Eukaryote (Protozoa)

All protozoans have a membrane-bound nucleus. They are nonfilamentous and inhabit moist or aquatic environments. They are found everywhere from South Pole to the North Pole. Cell size of protozoans ranges in size from 1 μm up to 2000 μm in diameter. They have both symbiotic and parasitic relationship. Protozoans are complex in their internal structure and therefore carry out complex metabolic processes. Protozoa have different structures which are used for their movement. For the process protozoans possess flagella, cilia or pseudopodia. Protozoans may have different shapes such as oval, spherical and elongated during their different phases of life cycle. One such stage is the trophozoite stage. This is the parasitic stage of protozoans that feed and reproduce vigorously, and when it has a tough membrane surrounding, it is known as cysts. They reproduce asexually by binary fission. All parasitic protozoans have holozoic mode of nutrition (depend on preformed organic substances). Protozoans are agriculturally important species. They feed on soil bacteria and enhance the fertility of soil. They also help bacteria in decomposing dead organic matter at a faster rate. Protozoans produce nitrogen as ammonium and phosphorus as orthophosphate as a metabolic byproduct, which helps in plant growth. Protozoans also have an indispensable role in wastewater treatment. Some commonly known protozoans include members like dinoflagellates, amoebas, paramecia and the malaria-causing *Plasmodium* (Diaz et al. 2019).

1.1.11 The Eukaryote (Viruses)

A virus is a miniature compilation of genetic code, either DNA or RNA, enclosed in a protein cover called capsid. It is submicroscopic particle not visible under a microscope. Their genetic material may be single or double stranded. A virus cannot reproduce on its own. It must infect cells and make use of replication system of the host cell it infects, to make copies of themselves. Often, in the process they kill the cell and cause harm to the host. An entire infectious virus particle is known as a virion. Viruses are everywhere. They have been discovered to be in greater abundance than other microorganisms (Lodish et al. 2000). Viruses are difficult to

eradicate. Antibiotics are ineffective against viruses because they lack the same machinery as bacteria; antiviral drugs or vaccinations, on the other hand, can eradicate or reduce the severity of viral infections, which includes AIDS, measles, smallpox, SARS-CoV (severe acute respiratory syndrome coronavirus), MERS-CoV (Middle East respiratory syndrome coronavirus), chikungunya virus, Ebola virus, Zika virus (Colson and Raoult 2016) and the present Covid-19. More details of virus are discussed in later section of this chapter.

1.2 How We Look at the Small World of Microbes

The use of light to observe the little and unseen world which otherwise is difficult to see with the human eye has become prominent in recent ages. The discovery of the microscope has been a breakthrough in this, to observe and study the different aspects of microbial cells. The use of microscopy techniques has enabled us to manipulate the different aspects of the cells like the cell colour and cell size that helps us to determine the specific type of an organism and also make a diagnosis about the diseases caused by them (Fleming 2019). To do so we need to have a clear understanding of the features that differentiate the prokaryotes from the eukaryotes. Also, this section travels to the period when and how various types of microscopes were discovered to use light as an insight into the invisible world of microbes. With the understanding of how different types of microscopes work, scientists can now explore microbes in depth by studying their images that may prove to be useful for both research work and clinical diagnosis. Microscopy and its working principles are better understood from their historical perspectives. Girolamo Fracastoro an Italian scholar, in his book *De contagione et contagiosis morbis* (1546), officially put forwards a postulate that diseases were caused by small invisible seminaria, or "seeds of the contagion", which could be transmitted through any possible types of contact (Iommi Echeverría 2010). However, during his time, technology for viewing tiny objects was not at an advanced stage; therefore the existence of the seminaria remained hypothetical for long—an invisible world remained unexplored till the discovery of microscope. In principle, it is believed that Girolamo Fracastoro was the first one to propose the germ theory even before Louis Pasteur and Robert Koch.

1.2.1 Difference Between Prokaryotes and Eukaryotes

The distinction between prokaryotes and eukaryotes gives us a clear picture of the different features in every group of organisms. Prokaryotes were the very first form of life on Earth. They appeared on Earth billions of years ago even before the eukaryotes came into existence. It is a well-accepted fact that differences in cell structural design between prokaryotes and eukaryotes are that prokaryotes do not possess membrane-bound organelles, whereas eukaryotes contain membrane-bound organelles including the nucleus (Fig. 1.3).

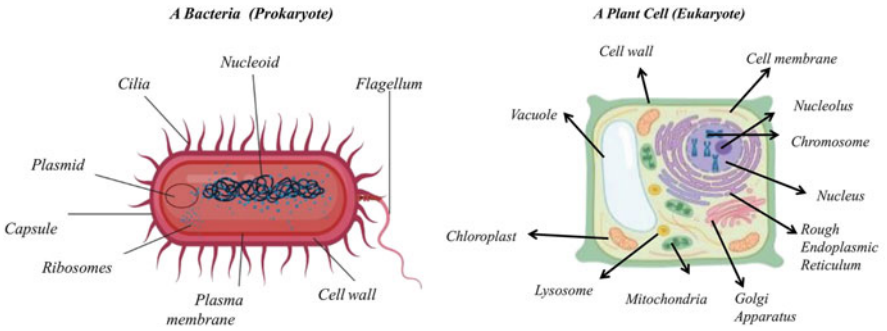

Fig. 1.3 Prokaryote and eukaryote

1.2.2 Microscopic Analysis of Microorganisms

Scientists made contributions in the field of microscopy to offer us images of small species that would otherwise have remained unknown, as described in the previous section. This may be seen in Robert Hooke's book Micrographia (1665), when he first described the structure of a cell by looking at a piece of cork under a compound microscope (Davidson 2010). We'll learn about the several types of microscopes used in the laboratory to investigate bacteria in this part.

1.2.2.1 Types of Microscopy

- *Light microscopy (bright-field microscopy)* is also popularly known as the compound light microscope. It is the basic microscope that is used in biology laboratories. It is an optical microscope that makes use of light rays to illuminate the specimen to produce a dark image against a bright background. This microscope is applied to observe fixed and live specimens, stained with basic stains producing a contrast between the image and the background. It has lenses that are modified versions of magnifying glasses that modify the specimen to produce an image to be seen through the eyepiece (Malacara 1988).
- *Darkfield microscopy* is used to create images of the live, unstained and transparent specimens which are high-contrast and high-resolution images. This microscopy offers an advantage as it uses live specimens which otherwise are killed by the use of stains. It is a bright-field microscope with a modified form of a condenser that makes use of cone-shaped hollow light. The condenser scatters light source causing it to reflect off the specimen at oblique angles making the specimen appear bright in contrast to a dark background (Omoto and Folwell 1999).
- *Phase-contrast microscopy* is an optical contrast method that allows unstained structures in biological specimens to be seen. It converts phase shifts in light to brightness in the image as it passes through the specimen. The structures of the specimen cause refraction and interference to create a high-contrast, high-resolution image of an unstained, transparent live sample. The image is formed by

altered wavelengths of light rays that pass through the specimen. For altering the wavelength of the light, an annular stop is fitted in the condenser. The annular stop produces a cone-shaped hollow light that is used to focus on the specimen. The organelles in eukaryotes and endospores in prokaryotes are best observed with phase-contrast microscopy (Zernike 1942; Barer 1947). Similar to phase-contrast microscopy, differential interference contrast microscopy (DICM) enhances the contrast of an image by using polarized light which converts phase shifts to light intensity changes. This produces an image of living organisms with a three-dimensional appearance making the technique useful in electrophysiological experiments. As an infrared (IR) light source of longer wavelength is generally used in DICM, therefore it is best suited for imaging thick specimens such as brain slices (Shribak et al. 2017).

- *Fluorescence microscopy* is an imaging technique. It visualizes the fluorescence emitted from the material to be analysed. The microscope transmits ultraviolet light of shorter wavelength, into the specimen; the fluorescent chromophores also called fluorochromes absorb the excited light and emit visible light with longer wavelengths that produces the image (Dallas 2020). The fluorochrome material is naturally occurring fluorescent substance, e.g. chlorophylls and fluorescent dyes which stain specimen creating the contrast. This technique is highly sensitive and provides information about the dynamic structure of polymers and nanomaterials where lights are adsorbed at liquid-liquid interfaces. Immunofluorescence, an important technique in fluorescence microscopy, is used to recognize pathogens by identifying antibodies bound to the pathogens if any (Sanderson et al. 2014).
- *Confocal microscopy* (also known as widefield fluorescence microscopy) views image by scanning and illuminating at different points of a sample using a pinhole (Rigby and Goldie 1999). It uses a laser light for fluorescence. It produces a high-resolution two-dimensional image which is converted to a three-dimensional image on a computer. This technique is frequently used in biomedical research to repair and fix damaged cells and to know about the various types of activities going inside a living cell. The confocal microscope has an advantage over other microscopes in that it can do thin optical sectioning of the tissues for viewing, and doesn't need any physical sectioning of the tissue (Rigby and Goldie 1999).
- *Two-photon fluorescence microscopy* is a breakthrough invention for imaging biological samples. It makes use of fluorochromes, infrared light of longer wavelength and scanning technique to visualize the specimens. The long-wavelength and low energy light allows photons to strike the same position in the specimen simultaneously exciting the fluorochrome. Viewing images with this microscope does less damage to biological specimens. The long-wavelength light examines the sample noninvasively from all dimensions with resolution less than a micrometre and enhanced diffusion depth. Two-photon microscopy is used to examine live cells in intact tissues such as the brain, embryos, complete organs and even animals (So et al. 2000; Helmchen and Denk 2005; Rosenegger et al. 2014).
- *Electron microscopy* (EM) An electron microscopy is a microscope that uses an accelerated electron beam as a light source. It allows researchers to view a

specimen as small as that of nanometre size. It uses short-wavelength electron beams with increased magnification and resolution (100,000×). EM is best used for viewing extremely minute characters of viruses, cell organelles, etc. (Savile Bradbury and Joy 2019). It is of two types:

- Transmission electron microscopy (TEM) is a technique for studying the characteristics of extremely small specimens. TEM uses a high accelerated ray of electrons that passes through a very thin sample and gives an in-depth internal view of the specimen. TEM makes use of electrons that have a shorter wavelength than photons, thus enabling the formation of an image of higher magnification and resolution.
- Scanning electron microscopy (SEM) creates an image by scanning a focussed electron beam across a surface. It is less powerful than TEM. SEM doesnot provide coloured image but creates black-and-white photographs of the specimen's surface (Golding et al. 2016).

- *Scanning probe microscopy* (SPM) is a relatively new method for examining structures at the molecular level. It is a conglomeration of related surface-sensitive techniques, applied to interrogate the specimen at an atomic scale with the aid of a sharp probe. This produces magnified images of up to 100,000,000 such that it allows viewing of every atom at an individual level. It uses no light source. It has the exceptional advantage, from a biological perspective. It not only allows high-resolution imaging of cellular structures below the optical limit but evaluates the micromechanical properties of the cell. It can examine cell dynamics and processes running even in real time in their most native physiological media such as aqueous solutions (Roberts et al. 1999).
  There are two types of scanning probe microscope:
  - *Scanning tunnelling microscope* (STM) produces an image by a fast-moving sharp metal wire tip over the surface of a specimen. The tip comes very near to the surface and applies an electrical voltage between the tip and the sample. The microscope scans every individual atom (Bottomley et al. 2005).
  - *Atomic force microscope* (AFM) works in the same way as STM. A sharp tip is scanned over a surface with a feedback loop to regulate the parameters desired to form the image of the surface. AFM uses atomic forces to record the interactions between the tip and surface of the sample to obtain the image (Bottomley et al. 2005).

1.3 Prokaryotes Diversity

The prokaryotes are the most abundant microorganism found on this Earth. Prokaryotes are the most diversified organisms, both metabolically and phylogenetically. Primarily prokaryotes were classified into single largest kingdom. However recent classification has divided prokaryotes into two categories, the bacteria and archaea, which believed to have evolved from distinct lineages (Oren

2004). Prokaryotic cells are small, single-celled and simple in architectural structure. Prokaryotic cells are surrounded by a plasma membrane and do not have membrane-bound organelles in their cytoplasm. And it is the absence of a nucleus and other membrane-bound organelles that differentiates prokaryotes from eukaryotes. Most prokaryotes contain a single chromosome bounded by a double-stranded circular DNA present in the nucleoid. The DNA in prokaryotes is present in the central area of the cell called the nucleoid. Prokaryotes have a circular DNA molecule called a plasmid, which is distinct from the chromosomal DNA and can replicate autonomously (Clark et al. 2019).

With the evolution going with time, archaea and the bacteria have split into many groups and species. Bacteria are divided into two major groups on their ability to retain stain: gram-positive bacteria and gram-negative bacteria. All the gram-positive bacteria belong to one phylum (Proteobacteria, Chlamydiae, Spirochaetes, Cyanobacteria), and the rest belong to gram-negative. The gram-staining method is named after its inventor, Danish scientist Hans Christian Gram (1853–1938) (Chinen et al. 2009). The property of different bacterial staining procedures is due to the presence or absence of a cell wall. Gram-positive bacteria lack the outer membrane, whereas the same is found in gram-negative bacteria. A major portion of the cell wall in gram-positive bacteria is composed of peptidoglycan layer, and the rest is made up of acidic substances called teichoic acid which covalently link the lipids in the plasma membrane to form the lipoteichoic acids. Lipoteichoic acids aid in anchoring the cell wall to the cell membrane. Gram-negative bacteria on the other hand have a thin cell wall composed of a few peptidoglycan layers bounded by an outer envelope of lipopolysaccharides (LPS) and lipoproteins.

The proteobacteria are further divided into alpha, beta, and epsilon groups. All of the animals in this category eat in a distinct way. Some members of the genus are photoautotrophic, some are symbiotic, and still others are human pathogens. *Rhizobium*, a nitrogen-fixing symbiont, *Rickettsia*, an internal obligatory parasite that causes diseases like typhus, *Helicobacter pylori*, which causes stomach ulcers, and *Salmonella typhi*, which causes food poisoning, are all members of this group.

The four other groups of bacteria are also diverse in their lifestyle (Ward 2002). Chlamydiae are pathogens that are intracellular obligate parasites found in animals causing sexually transmitted disease, e.g. *Chlamydia trachomatis*, while Cyanobacteria are photosynthetic bacteria, e.g. *Prochlorococcus*, that contribute half of the oxygen available on this planet. Spirochaetes include both free-living anaerobes which are harmless in nature and pathogenic ones, like the *Treponema pallidum* causative agent of syphilis. The gram-positive bacteria are also not behind in contributing to both health and diseases. They include antibiotic-producing bacteria *Streptomycin*, probiotic bacteria such as *Lactobacillus* sp. present in yogurt and *Bacillus anthracis* causing the disease anthrax.

1.3.1 Archaea

Archaea are single-celled creatures that belong to the Archaea domain. Like prokaryotic cells, they also do not have cell nuclei. Archaeal cells have distinct characteristics that distinguish them from Bacteria and Eukaryota. Archaea are further subdivided into several distinct phyla. Euryarchaeota, Crenarchaeota, Nanoarchaeota, and Korarchaeota are the four primary groups in the domain archaea. Among them the Crenarchaeota and Euryarchaeota are the two phyla that have been examined the most. Methanogens, halobacteria, Sulfolobus, and Nanoarchaeum equitans are all members of this group. Till now there are no reports of the discovery of archaea as human pathogens. Classifying the archaea is somewhat difficult, since the majority of its phylum has never been studied and has mainly been identified by nucleic acid sequencing of the samples from the environment. Archaea mostly inhabit the gut of the humans and animals and all seem to be harmless and render more benefits to the gut. The archaea are also extremophile species; they can live in inhospitable conditions such as radioactive environment, high saline aquatic bodies, etc. Archaea are thought to be significant in global biogeochemical cycling, since they comprise a probable 20% of the world's total biomass. Archaea reproduce asexually by binary fission (Michael Hogan and Monosson 2010) (Fig. 1.4).

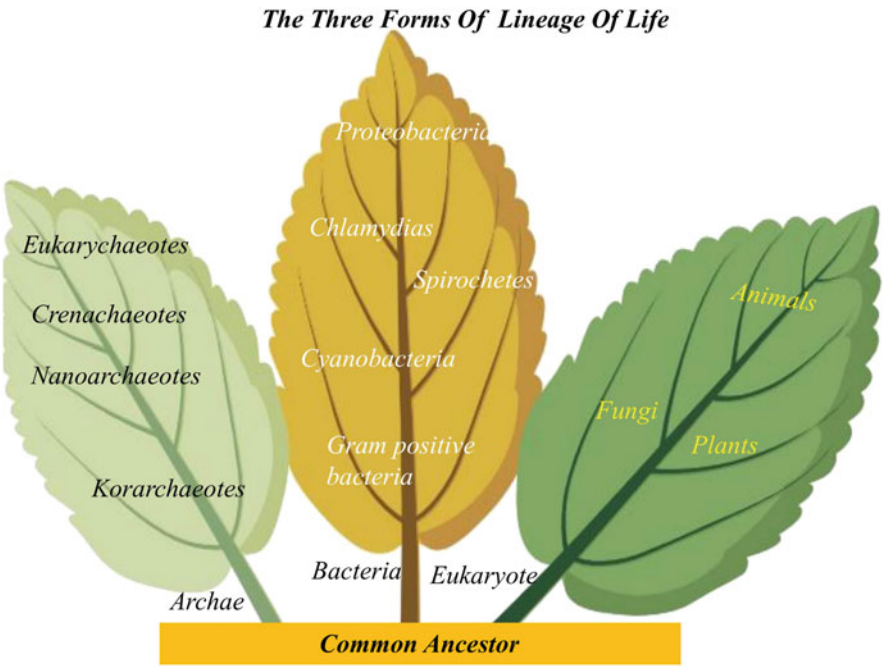

Fig. 1.4 Showing the lineages of three forms of life from a common ancestor. (Adopted and modified from *OpenStax College*, *Biology*)

1.4 Viruses, Viroids, Virusoids and Prions

The virus is a submicroscopic particle that is too small to be seen by an ordinary light microscope. Viruses infect all forms of life, which include animals, plants and microorganisms, such as bacteria and archaea (Koonin et al. 2006). Viruses use the replication machinery of the host cell to make copies of themselves (Lodish et al. 2000). In the process, they kill the host cells and cause serious damage to the host organism. Viruses are found everywhere on Earth (Edwards and Rohwer 2005; Lawrence et al. 2009). Viral diseases have been here on this planet since the onset of life but were not studied until the twentieth century. In 1886, the German chemist Adolf Mayer showed that tobacco mosaic disease (TMD) was an infectious agent and could be transmitted from a diseased plant to a healthy plant. Following this discovery, the Russian bacteriologist Dmitrii Iwanowski (1892) made an effort to isolate the agent of TMD (Artenstein 2012). He observed that the sap of diseased plants which he filtered using his self-designed porcelain filter to retain bacteria had instead filtered out through the tiny pores of the filter. Hence, it was Martinus Beijerinck in 1898 who finally reported the tobacco mosaic virus (Kammen 1999). Since then around 200,000 virus species have been reported (de Jesus 2019). Viruses are an interesting organism. They are made up of two or three components. They are a minute aggregation of either single-stranded or double-stranded RNA or DNA, enveloped by a protein coat capsid (Gelderblom 1996). Viruses are a different entity and don't have the same components as bacteria, so any antibiotics are ineffective against them except antiviral drugs or vaccines which can eradicate or reduce the severity of viral diseases already discussed earlier (Graham 2020). Viral diseases such as measles and smallpox have been completely eradicated, yet research is in full swing to find complete cure for diseases like AIDS, Covid-19, etc. Advancement in the biological science in the later part of the nineteenth century led to the identification of several new virus causing diseases such as human immunodeficiency virus (HIV), SARS, MERS-associated coronavirus, H5N1 influenza A and H1N1 to name a few (Abdelrahman et al. 2020). Outbreak of diseases, such as Israeli acute paralysis virus (2006) (Chen et al. 2014), Ebola virus (2018) of Eastern Democratic Republic of the Congo (Aruna et al. 2019) and Nipah (2018) in India (Thomas et al. 2019), was also reported which had become a national concern in each of these countries. The different types of viruses are represented in Fig. 1.5.

Viroids

In 1971, pathologist Theodor Diener, at the Agricultural Research Service, isolated an agent from diseased higher plants, which lacks the property of an intact cell particle that he named as viroid, meaning "virus-like". Viroids are the smallest pathogens known, single-stranded non-coding RNA molecules that do not code protein yet reproduce independently via rolling-circle mechanism when introduced into host plants (Owens 2008). Viriod consists of low molecular weight RNA. Viroids are tiny pathogens that cause disease. They are made up entirely of a single-stranded circular RNA strand. They do not have a protein covering, unlike viruses. They have a short genome size, which protects them from disaster caused by

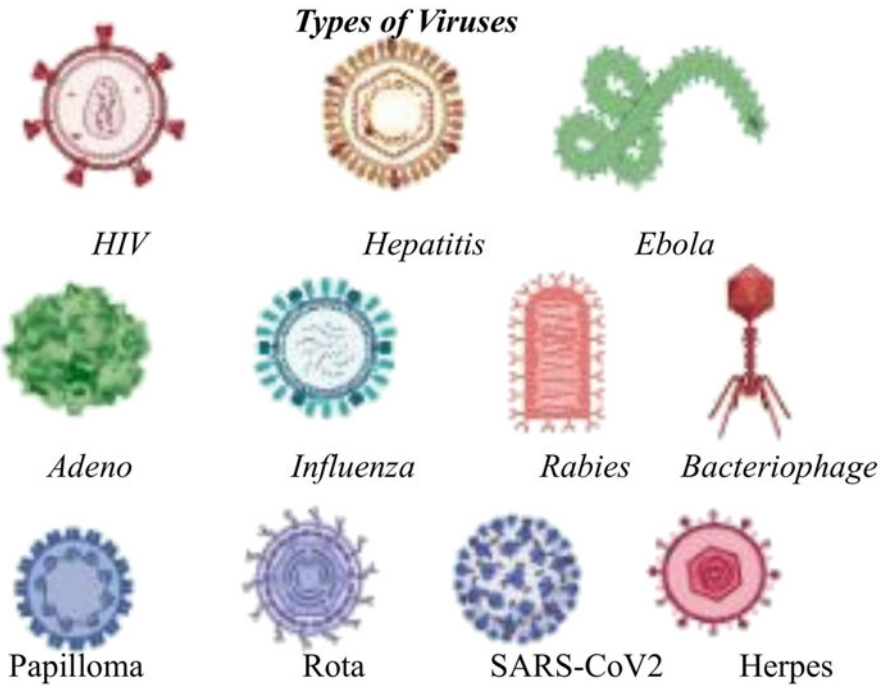

Fig. 1.5 Schematic representation of different types of viruses

error-prone replication, a high GC content, which provides thermodynamic stability, and circular genomes, which prevent information loss. Some viroids are ribozymes, which have catalytic capabilities that allow unit-size genomes to be self-cleaved and ligated from bigger replication intermediates. Viroids reproduce independently with the help of the host's machinery (Kovalskaya and Hammond 2014). The viroid was found to cause the disease potato spindle tuber viroid, which leads to late sprouting and various defects in potato plants. Viroids can be found in monocot and dicot plants, herbs and woody plants, agronomically and ornamentally important plants. Viroids are globally distributed, and their distribution is attributed to the exchange of infected germplasm and transmission through seed and insects. This has resulted in scarcity of food availability and collapsing of the economy (Hadidi et al. 2003). Other diseases caused by viroids include tomato planta macho viroid (TPMVd) in tomato, avocado sunblotch viroid in avocado, peach latent mosaic viroid in peach, *chrysanthemum stunt viroid* (CSVd) in chrysanthemum, *citrus exocortis viroid* (CEVd) in citrus, *coconut cadang-cadang viroid* (CCCVd) in coconut palm and *avocado sunblotch viroid* (ASBVd) in avocado, to name a few (Diener 1987; Hadidi et al. 2003) which lead to loss of chlorophyll content, fragile leaves and small-sized fruits, causing fungal and bacterial infection ultimately resulting in loss of productivity in these field crop. At present around 34 viroid species have been recognized,

length ranging from 120 to 475 nucleotides, and are categorized into two families, *Avsunviroidae* and *Pospiviroidae* (Di Serio et al. 2014). The members of *Avsunviroidae* (*avocado sunblotch viroid*) replicate in the chloroplasts or plastids via symmetric rolling-circle replication using the nuclear polymerase enzyme of the host. Members of *Pospiviroidae* (*potato spindle tuber viroid*) replicate in the nucleus via asymmetric rolling-circle replication using RNA polymerase II (Pol II) enzyme of the host. Their RNAs are rod-shaped having secondary structures without the ribozyme activity (Flores et al. 2014; Ding 2009). The exact mechanism of viriod infection is not fully understood, although identification of viroid-infected plants is accomplished by either symptomatology on indicator hosts, a classic method still used in many certification programmes, or molecular methods such as nucleic acid hybridization or polymerase chain reaction. Flores et al. 2015; Hammond and Owens 2006).

Virusoids

Virusoids are subviral particles, which are part of a specific group of satellite RNAs, connected with the sobemoviruses, and share several common structural and functional characters with viroids. The genome of the virusoid is small, having about 246–371 nucleotides long (Shrestha and Bujarski 2020). Only the satellite RNA of virusoid codes for protein and replicates via rolling-circle mechanisms. Despite these similarities, virusoids differ in certain features from viroids. Virusoids replicate in the cytoplasm with the help of both helper viruses and hosts (Ding 2010). Also, virusoids are coated by their helper virus coat proteins. One example of helper virus is the hepatitis B virus (HBV) found in humans to infect liver along with the satellite RNA hepatitis delta virus (HDV) or hepatitis delta virusoid (Taylor 2015), and the other found in plants is the subterranean clover mottle virus. Once the helper virus enters the host cell, the virusoids are released to be set free in the cytoplasm of the plant cell, having ribozyme activity (Francki et al. 1983). HDV is much larger (1700 nucleotides) than a plant virusoid, with a circular, ssRNA genome, and actively involved in the biosynthesis of HDV-associated proteins (Taylor and Pelchat 2010). There are reports that viroid RNAs are involved in trafficking activity intracellularly, replication and infection (Ding and Itaya 2007). The biological functions of virusoids remain vague.

Prions

Stanley Prusiner Nobel Prize-winning biologist discovered prions or cellular prion protein (CPrP). It is a glycoprotein of mammals which is found attached to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor (Sarnataro et al. 2017). It is smaller in size compared to virus, with no nucleic acids (neither DNA nor RNA) (López-Pérez et al. 2020). The molecular structure of CPrP has been determined by nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography. The normal prion protein is made up of mutated alpha helices, and beta sheets (Kupfer et al. 2009). Prion-like features have been found in some types of fungi. These fungal prions have been used to study how prions causes infection (Wickner et al. 2007). Prions cannot be destroyed normally as other pathogens as

they do not have nucleic acid. Since prions are the mutated form of a normal protein that is encoded in the body, they do not trigger a host immune response, as other pathogens do (Zabel and Reid 2015). Cellular prion protein is present on the cell surface of the central nervous system, and also in other tissues in the body (Wulf et al. 2017). A prion is responsible for progressive neurodegenerative conditions. The names of two such diseases are bovine spongiform encephalopathy (BSE or mad cow disease) found in cattle and other farm animals and kuru in humans. These misfolded proteins do not infect by replication in the host. Instead, they behave as an inducer adversely affecting the brain by changing the structure of normally folded proteins to the abnormal prion form ($CPrP^{SC}$), which further acts as a template accumulating the abnormal prions in the tissue of the central nervous system. They develop plaques which lead to cluttering of neurofibrils and interfere with the functioning of the synapse. As a result of the process, the nerve cells are damaged and forms tiny pores in the brain, thus giving it a spongy appearance under the microscope and the name spongiform disease (Cobb and Surewicz 2009). Kuru was found to originate in humans in Papua New Guinea, transmitted to humans by means of ritualistic cannibalism. BSE was first discovered in the United Kingdom, by including cattle nervous tissue in the feeds for other cattle which had led to the spread among other cattle. Patients suffering from kuru and BSE showed clinical symptoms like failure of motor function and behaving abnormally such as gelastic epilepsy (sudden burst of laughter) ultimately causing death. Another disease was discovered during an outbreak of BSE, a similar type of encephalopathy in humans known as variant Creutzfeldt-Jakob disease (CJD) which had spread due to consuming beef meat from animals infected with BSE (Cobb and Surewicz 2009). The disease is transmitted via blood transfusion among humans. There are other prion disease that might develop in the complete absence of infectious $CPrP^{sc}$, suggesting the presence of a transmembrane form of the protein tmCPrP which is inherited from the human prion disease (Kovacs and Budka 2008). The different structure of prion mutants and the presence of the GPI anchor have immense power both on the function and the intracellular transport of the prion protein which ultimately causes the disease (Puig et al. 2019).

1.4.1 Classification of Virus

Bennett proposed the first systematic classification of viruses in 1939 on behalf of the newly formed Committee for Virus Nomenclature of the Council of the American Phytopathological Society (Kuhn 2021). Viruses were classified according to the type of morphology and the disease they cause in their hosts, their host tropisms, their modes of transmission and the antigenic, chemical and physical properties of their particles. Bawden, in 1941, suggested a classification based solely on chemical, antigenic and morphological properties of virus particles which was followed by others for many years. In 1948, Francis O. Holmes proposed systematic taxonomic classification that was used for animals and plants. He suggested a top-rank taxon, the order "Virales", that would include three suborders for viruses that infect bacteria

("Phaginae"), plants ("Phytophaginae") or animals ("Zoophaginae"). However, this system did not gain much popularity. There were many classifications put forwards by several taxonomists, but ultimately the taxonomic classification that received broad attention is that of Lwoff, Horne and Tournier (the LHT system), which got approval in the year 1966. The LHT system classified viruses into one phylum ("Vira") with two subphyla based on the presence of RNA or DNA in virions ("Deoxyvira" and "Ribovira"). Each subphylum was further classified based on the symmetry of virion capsids (e.g. the classes "Deoxyhelica" for "helical DNA viruses" and "Ribocubica" for "cubical RNA viruses"). These classes were further classified into orders ("virales") with suborders ("viridales"), families ("viridae"), subfamilies ("virinae"), genera ("virus") and subgenera ("virus") and described species type and common names for taxon members. Even though categorization of the higher taxa was outdated, several family names are still being used today (e.g. "Poxviridae", "Adenoviridae" and "Paramyxoviridae" became the present Poxviridae, Adenoviridae and Paramyxoviridae, respectively), along with the suffixes for order, family, subfamily, genus and subgenus names, which are now italicized ("virales", "viridae", " virinae", "virus"). Viruses are classified on the basis of morphology, chemical composition and mode of replication. The viruses are classified according to the infection they cause in humans. They are at present grouped into different number families, whose host ranges from vertebrates to protozoa and from plants and fungi to bacteria (Gelderblom 1996). Here we will discuss the classification of virus on different aspects.

1.4.1.1 Morphology: Helical Symmetry and Icosahedral Symmetry

Every virus has a protective layer of protein capsid to give protection to its nucleic acid genome. Viral capsids are of two kinds: helical and icosahedral (Louten 2016). During replication of virus with helical symmetry protomers, the protein structural subunits self-assemble into a helical arrangement covering the nucleic acid, forming a spiral shape that twists cylindrically around an axis. In the case of a helical virus, the viral nucleic acid coils into a helical shape, and the capsid proteins wind around the inside or outside of the nucleic acid, forming a long tubular or rod-like structure (Gelderblom 1996). The nucleic acid and capsid together constitute the nucleocapsid which form stiff, highly extended rods and flexible filaments. When inside the cell, the helical nucleocapsid uncoils and the nucleic acid is released making itself accessible. Viruses of the families Paramyxoviridae, Orthomyxoviridae (Couch 1996) and Coronaviridae show helical symmetry. The most extensively studied helical virus is tobacco mosaic virus. Of the two capsid structures, the icosahedron is the most widespread than the helical viral structure. An icosahedron is a polyhedron having 20 equilateral triangle with 12 vertices or faces. An icosahedron has 2-3-5 symmetry (which implies it has twofold, threefold and fivefold axes of symmetry).

1.4.1.2 Morphology: Complex Viral Structure

Viruses have a symmetry coated with a proteinaceous compound called capsid. This capsid has many roles. These include virus attachment to cells, entry into cells,

release of capsid contents into cells, and packaging of newly generated viral particles are all roles played by the capsid during viral infection. The capsid is also in charge of viral genetic material transfer from one cell to another. These structures also influence the viral particle's stability, such as resistance to chemical or physical inactivation (San Martín 2013). Complex viruses create a range of symmetrical mismatches in icosahedral shells to completely asymmetric or pleomorphic capsids (San Martín 2013). This structural complexity of the virus is due to large size genome for larger capsid is the requirement. They may also contain accessory proteins with specific structural or functional roles. For example, HIV have genome size of 7–10 kb of single-stranded (ss) RNA (Goff 2007). Examples of other complex viruses are poxviruses, geminiviruses and many bacteriophages. Poxviruses include the viruses that cause smallpox or cowpox. They are large oval or brick-shaped particles sized 200–400 nm long. Bacteriophages, also known as bacterial viruses or prokaryotic viruses, are viruses that infect and reproduce within bacteria (Louten 2016). The viral structure based on morphology is depicted in Fig. 1.6.

1.4.1.3 Morphology: Presence and Absence of Envelope

Some virus families have an additional extra layer, termed as envelope, which is the extra growth of the modified cell membrane of the host. This envelope is composed of lipid bilayer whose exterior is embedded with virus-encoded, glycosylated (trans)membrane proteins which are like spikes or knobs also known as peplomers. These glycosylated proteins determine the host range and antigenic property of the virion. Virus envelopes also provide an additional protective coat (Firquet et al. 2015).

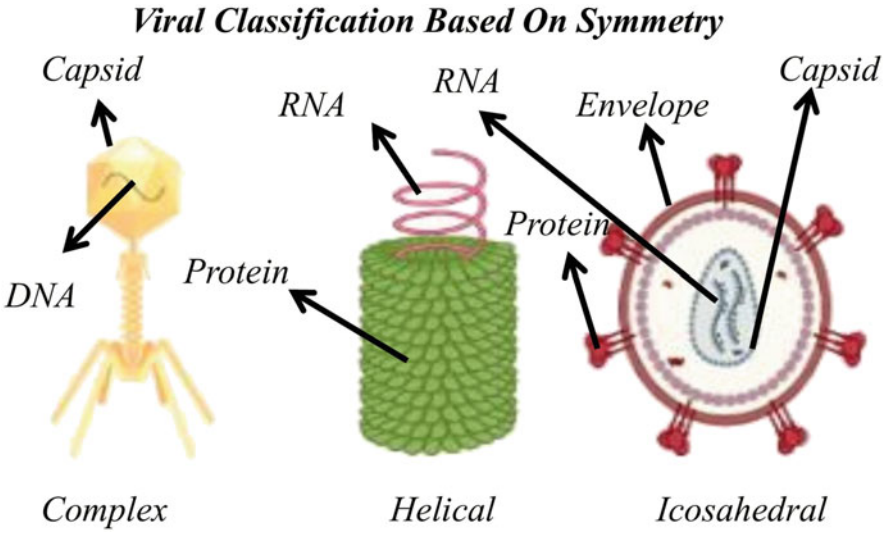

Fig. 1.6 Schematic representation of viral symmetry

- Some members of enveloped virus include the following:
  Influenza virus type A (H1N1), Herpes Simplex Virus Type 1 (HSV-1), Human Coronavirus
- Some members of non-enveloped virus include:
  Coxsackieviruses, Rotavirus, Poliovirus

1.4.1.4 Chemical Composition and Mode of Replication

Viruses are submicroscopic species considered to be non-living particles which cannot replicate independently outside the living host. A virus consists of either DNA or RNA as their genetic material covered by a protein coat. The genome of a virus may contain DNA or RNA, which may be single stranded (ss) or double stranded (ds) or linear or circular. The entire genome is either unsegmented/monopartite (having one nucleic acid molecule) or segmented/multipartite genome (several nucleic acid segments). It is therefore this variety of genome that leads to different replication strategies. Accordingly, they are classified as DNA viruses and RNA viruses and according to mode of replication.

DNA Viruses

DNA viruses have DNA genomes that are replicated inside the host with the help of its DNA polymerases and deoxynucleotide triphosphate precursors. It is larger than RNA virus. DNA viruses are divided into three major categories: double-stranded DNA viruses (e.g. poxviruses), single-stranded DNA viruses (e.g. parvoviruses) and pararetroviruses (e.g. hepadnaviruses) (Kovalchuk and Kovalchuk 2016). DNA viruses mostly contain a single genome of linear dsDNA. dsDNA serves as a template both for mRNA and for self-transcription process (Payne 2018). DNA viruses have different replication system. They need prior de novo synthesis of proteins to provide the protein products needed for DNA replication. These are generally called "early genes" (White and Spector 2007). After DNA replication, forms the expression proteins, they are required to be packed which later form virions. These are generally called "late genes". DNA viruses consist of important pathogens such as herpesviruses, smallpox viruses, adenoviruses and papilloma viruses, among others.

RNA Viruses

RNA viruses have RNA as their genetic material. It may be a single-stranded RNA or a double-stranded RNA and uses RNA-encoded RNA polymerases for replication of their genomes. Retroviruses, on the other hand with two copies of single-stranded RNA genomes, use reverse transcriptase which produces viral DNA that can be integrated into the host DNA using its integrase function (Poltronieri et al. 2015). During replication of RNA viruses, three types of RNA must be formed: the genome, a copy of the genome (copy genome) and mRNAs. The RNA polymerases and other proteins required for the synthesis of viral genome are called the replicase complex. The replicase complex comprises the set of proteins required to produce infectious genomes. The RNA virus is subdivided based on the type of RNA they contain. Positive- or plus (+)-strand RNA viruses are those whose genomes have functional

mRNAs that are translated to produce RNA-dependent RNA polymerase which in turn is used to produce RNA virus. The other three groups of RNA viruses are those whose genomes are not mRNAs. They are the negative- or minus-strand RNA viruses, the closely related ambisense RNA viruses and double-stranded RNA viruses (Payne 2017). Human diseases caused by RNA viruses include orthomyxoviruses, hepatitis C virus (HCV), Ebola disease, SARS, influenza, polio, measles and retrovirus including adult human T-cell lymphotropic virus type 1 (HTLV-1) and human immunodeficiency virus (HIV) (Poltronieri et al. 2015).

DNA-RNA Viruses

The RNA tumour viruses called leukoviruses and Rous Sarcoma Virus unusually contain both DNA and RNA as genetic material (Bader et al. 1970).

ICTV Classification

As viruses are non-living entities, they are classified using order, family, genus and species unlike living organisms which are classified using domain, kingdom, phylum, class, order, family, genus and species taxa. Therefore to deal with the challenge, the International Committee on Taxonomy of Viruses (ICTV) was formed and has been responsible for classifying viruses since 1966 (Louten 2016). ICTV utilizes taxonomical system of classifying the viruses. ICTV while classifying viruses takes into consideration the size of the virus, capsid structure, type of nucleic acid, physical properties, host species or disease. A complete catalog of known viruses is maintained by the ICTV at ICTV database known as Virus Metadata Repository. The first ICTV report included only 2 families, 27 genera, 10 subgenera and 18 "virus groups", but the classification system has been extended since then. For instance, the first order (*Mononegavirales*) was established in 1991, and in 2019 the first phylum (*Negarnaviricota*) and realm (*Riboviria*) were made official. *Riboviria* contains nearly all RNA viruses and reverse-transcribing viruses; three separate proposals were approved to establish three realms for viruses with DNA genomes (Walker et al. 2020). By that time, 5560 species had been established (Kuhn 2021). The latest report on containing updated data on new species ratified can be accessed in Virus Metadata Repository: version August 1, 2020; MSL35 (https://talk.ictvonline.org/taxonomy/vmr/m/vmr-file-repository/10312).

Baltimore Classification (1971)

Nobel laureate David Baltimore proposed a classification of virus in the early 1970s that is still today parallelly used with official virus taxonomy. Rather than grouping the viruses to taxa, Baltimore grouped all viruses into six disconnected groups without any subdivisions, now commonly referred to as Baltimore classes (BCs). The Baltimore classification system classifies viruses based on the type of nucleic acid genome they have and replication system of the virus. The system also divides single-stranded RNA viruses into two groups: those that have positive strand (+) and negative strand (−). Positive-strand (also positive-sense or plus-strand) RNA virus is the one which translates into proteins, such as messenger RNA (mRNA) in the cell is positive strand. Negative-strand (also negative-sense or minus-strand) RNA virus is

the one which is not directly translated into proteins. It is first transcribed into positive-strand RNA. Baltimore also included viruses that are able to do reverse transcription, or form DNA from an RNA template, which is something that cells are incapable of doing. The seven classes of Baltimore classification are as follows:

- BC I: double-stranded DNA genome virus that has a replication-expression strategy highly suggestive for that of cellular organisms (e.g. *Adenoviridae*)
- BC II single-stranded DNA genome virus (e.g. *Geminiviridae*)
- BC III: double-stranded RNA genome virus (e.g. *Reoviridae*)
- BC IV: positive-sense RNA genome virus (e.g. *Picornaviridae*)
- BC V: negative-sense RNA genome virus (e.g. *Paramyxoviridae*)
- BC VI: positive-sense RNA genome virus that replicates using DNA intermediates produced by reverse transcription of the genome (e.g. *Retroviridae*)
- BC VII (which was added later to the original system): double-stranded DNA genome virus that packages a double-stranded DNA form or an RNA-DNA hybrid into virions and replicates via reverse transcription (e.g. *Hepadnaviridae*)

1.5 Tools and Techniques of Microbiology

Microbes are universal, and to have a better knowledge of microbes, it is important to know the laboratory and the experiments carried out. Our present knowledge of physiology and regulatory activity of microorganisms has arisen from our ability to isolate them from their natural ecosystems into pure form in the laboratory cultures (Palková 2004). The microbial techniques used in the laboratory are the ones used to study microbes such as bacteria, fungi and protists. They incorporate techniques such as surveys, isolation, culture and manipulation of microorganisms. The first artificial liquid culture medium was developed by Louis Pasteur in 1860 (Bonnet et al. 2020). The study of microbiological tools and techniques is essential as microbes are an important part of our life and are both beneficial and harmful. The present-day threat of an outbreak of pandemics or use of bioweapon in warfare led to public consciousness towards the importance of applying proven precautionary procedures and healing (Riedel 2004). Laboratory culture remains an indispensable part of science. The research work carried out provides a solution towards the issues in society related to health and diseases. Microbiology techniques are very useful in agriculture, evolutionary studies, pharmaceutical industry (Vitorino and Bessa 2017). Modern-day use of biotechnology tools and techniques has encouraged the use of new methods to make further advances in microbiology as a scientific discipline. Microbes are present everywhere around us. To identify and purify specific microbes, they have to be cultured in a controlled and optimized laboratory setup in aseptic conditions. Aseptic techniques are performed under sterile conditions, a method that prevents the entry of contaminants into the working space. This process is executed by strictly adhering to guidelines laid down in the laboratory manual. Culture techniques were developed from the need to grow and identify potential infectious agents for the diagnosis and treatment of any diseases.

Before going into the details of microbial culture techniques, let us first discuss about some basic technique requirements.

- Growth medium is a nutrient medium where microorganisms grow in any type of organic compound that contains carbon and nitrogen in the nutrient medium. Carbon is used for respiration and nitrogen is used for growth. Media can be both solid and liquid. Depending on growth, nutrient media may be enriched with minerals and vitamins.
- The killing or elimination of all microorganisms is known as sterilization. Sterilization can be done using both physical and chemical methods. Physical methods make use of heat, radiation and filtration. To validate the sterilization result via chemical and physical methods, biological indicators may be used. The sterilization processes should be done under strict control measures to get the appropriate results as the frequency of resistance varies. It ranges from highest in the case of endospores to moderate to least resistance in the case of vegetative cells. The most frequently used instrument in the laboratory for the sterilization of media and glassware is the autoclave.
- *Disinfectants* are chemical agents applied to non-living objects to kill microbes except endospores. *Antiseptics* are antimicrobial substances that are applied to living tissue to decrease the prospect of an infection. Disinfectants are of two types—bactericidal one that kills the bacteria and bacteriostatic that hinders the growth of the cell and reproduction.
- Sanitization is done by using a cleansing technique by removing microbes to reduce the contamination. The sanitizers are soap or detergent.
- Inoculation is the technique by which microorganism is introduced into the media.
- Incubation is the process of keeping the inoculated media in an incubator for the growth of the organism under optimal temperature and pressure.

1.5.1 Isolation of Microorganisms

Microorganisms occur in the natural environment in association with each other. Among them some are pathogenic and some are non-pathogenic. The pathogenic ones might cause lethal diseases. Therefore it is necessary to isolate and culture them in pure form according to their functional properties, to identify and control the infectious agents. The primary culture from a natural source will normally be a mixed culture containing microbes of different kinds. In the laboratory, these mixed cultures may be isolated into single or pure culture.

1.5.1.1 Methods of Isolation

There are many specialized techniques used to get pure cultures of microorganisms. A pure culture contains a single bacterial species. An isolated pure culture may be obtained using enriched media containing definite chemical or physical agent components that allows the isolation of the target organism. The basic methods for

isolation of a pure culture include (1) spread plating on solid agar medium using a glass spreader and (2) streaking with a metal loop. The purpose of these two techniques is to isolate individual bacterial cells (colony-forming units) on a culture medium. Both procedures should be executed aseptically (Mara and Horan 2014; Sanders 2012).

- *Streaking*: The streaking technique isolates individual bacterial cells on the surface of an agar plate with the help of a metal wire loop. It is sterilizable and can be reused. While working with Bio-safety Level-2 microbes in a biosafety cabinet, disposable plastic loops may be used. Some scientists may prefer using disposable, presterilized wooden sticks for streaking. The inoculum containing the mixed bacterial culture is spread evenly over the semi-solid surface of agar-based nutrient medium in a Petri dish in a zigzag/quadrant pattern followed with incubation, the inoculum which later grows into visible and viable colonies. The inoculums may be taken either from a suspension of cells (5–10 μL with a micropipette) in broth or from existing viable colonies from another agar plate. The cell suspension is vortexed before applying the aliquot for plating. All the steps are to be performed aseptically.
- *Pour plate procedure* (enumeration of bacterial cells in a mixed sample): This method is used to count the number of viable bacterial cells present in a mixed culture. The mixed culture is poured on a molten agar medium before its solidification (cooled at 4 °C). It includes dilution of the mixture of microorganisms in the broth until only a few hundred bacterial cells are left in each millilitre of the suspension/broth. A very little amount of the diluted inoculum is then placed in a sterile Petri plate employing a sterile loop or pipette. The microorganism and agar are properly mixed and incubated till the appearance of the visible colonies. Viable plate count allows scientists to generate growth curves, to calculate the cell concentration.
- *Spread plate procedure* (formation of discrete bacterial colonies for plate counts): This technique is used to isolate microorganisms when the volume of the sample in the inoculum is less. The inoculum is added to the agar plate with the aid of a wire loop or a micropipette which is then spread over the agar surface using a spreader. Separate colonies grow uniformly distributed across the agar surface. The advantage of this experiment is that it not just allows the growth of viable cells but also allows to examine the growth of cells of a particular genotype. This technique is used for the enumeration of viable cell count. There are two types of spread plate methods. The first one is the turntable method where a glass or metal rod is used. The second method is known as the "Copacabana method", which involves shaking the culture media with presterilized glass beads. Both ensure the smooth spreading of cells across the agar surface. There are other techniques used for the enumeration of viable cell count. They are discussed as follows:
  - *Enrichment culture method*: This involves culturing of mixed organisms in conditions that favour the growth of desired microorganisms with desired metabolic properties and activity. This culture also allows the growth of

other undesired organisms simultaneously. The colonies that grow show phenotypic properties along with the desired genotype.
  - *Selection experiment*: This allows the growth of cells with a particular type of genotype. This type of experiment is done in molecular biology laboratory where recombinant cells are cultured. For example, bacterial strains with plasmids containing antibiotic-resistance genes when plated on a medium supplemented with a particular concentration of an antibiotic will allow selection for those cells that have resistance to that particular antibiotic.
  - *Screening experiment*: In this experiment mixed culture is plated on a medium that allows the growth of those viable cells with the desired genotype expressing a particular phenotype. This experiment again is prevalent in molecular biology laboratories where cloning experiments are performed. One such example is the blue-white screening technique for the recognition of recombinant bacteria. The activity is performed by β-galactosidase, an enzyme occurring in *E. coli*, which cleaves lactose into glucose and galactose.
- *Soft agar overlay procedure* (formation of plaques for isolation and enumeration of phage (plaque assay)): This method is used to identify and quantify microbes and bacteriophage (phage) in culture. Although an electron microscope is employed to observe individual phage particles, they can be detected by the formation of plaques on an agar plate. The phages cannot replicate independently outside their host bacterial cells, so their multiplication and identification require mixing of both the phage suspension and host cells (bacteria) in a test tube before inoculating in the plate. The resulting mixture is poured over the surface of a nutrient agar plate and then incubated. If the phage infects the bacteria, plaques appear which can be observed either by the naked eye or by microscopy. Each plaque is a single virus or phage, and the number of plaque-forming units (pfu) may be calculated, and the concentration, of the phage suspension, may be determined. This experiment, also known as plaque assay, may be used to generate growth curves and for converting bacterial cells for experiments in genetics.
- *Replica plate procedure* (transfer of cells for screening mutants and auxotrophs): Replica plating is a microbiological approach during which one or more secondary Petri plates containing selective growth solid media (missing nutrients or growth inhibitors like antibiotics) are inoculated with an equivalent colonies of microorganisms from the primary plate (or master dish), reproducing the first spatial pattern of colonies. The technique involves producing the exact copies of the colonies within the same spatial pattern in a secondary plate by simply pressing a small piece of velvet to the first. This system is advantageous because it allows a comparatively sizable amount of colonies to be screened simultaneously for several phenotypes during a single experiment.

1.5.2 Types of Nutritional Requirements and Media

Microbes can be categorized based on how they obtain carbon, energy and electrons or hydrogen. Undeniably, the exact nutritional requirements of microorganisms are used to differentiate microbes from another for taxonomic classification. Microorganisms form groups on the basis of their energy needs. Microbes that obtain energy by oxidizing chemical compounds (either organic or inorganic) are called *chemotrophs*, and those that use light energy are called phototrophs (Chan 2003). Therefore to mimic the natural environment in the laboratory to grow the microbes, the culture media were used. Culture media are the foundation of microbiological tests. They are applied to isolate and cultivate pure microbial cultures. A microbiological culture is the medium for a microbe to grow and survive from where it acquires nutrients, factors that promote growth, buffer salts, minerals and gelling agent. Culture media have been in use since the onset of the nineteenth century. The first culture medium prepared was meat-infusion broth by Robert Koch and his colleagues (Sandle 2010). Even with the technological development, majority of techniques used in microbiology laboratory require growth culture media. Such is the importance to have a basic understanding and knowledge of culture media (Bridson and Brecker 1970; Sandle 2010). Media can be differentiated in line with the content material of the nutrients and consistency and may be classified consistent with their physical nature, chemical composition and the function they perform.

1.5.2.1 Classification of Media on Physical State

- Liquid media are useful in the isolation of small numbers of microorganisms. It has no solidifying agent added, for example, nutrient broth.
- Solid media are defined as the media formed by adding of agar to the culture media as a solidifying agent. The concentration of agar can be changed accordingly to make the medium solid or semi-solid. Nutrient agar prepared by adding 2% agar to nutrient broth forms the solid medium. Examples of solid media include blood agar, chocolate agar, MacConkey agar, etc.
- Semi-solid media use less concentration of agar (0.2–0.4%) to make the medium semi-solid.

1.5.2.2 Classification of Media on Composition

- Complex media are undefined and non-synthetic. In undefined media chemicals present are in unknown proportions. Examples of complex media are chocolate agar and MacConkey agar.
- Chemically defined medium is synthetic, containing known quantities of the ingredients. It contains no natural ingredient. Dubos medium with Tween 80 is an example of this medium.

1.5.2.3 Classification of Media on Function

- Enriched media are solid in consistency. It allows fastidious bacteria (organisms that are incapable to synthesize certain nutrients on their own and have to be

supplemented to the medium) to grow. These media are prepared by adding blood, serum and egg to the basal media (Bonnet et al. 2020).

- Enrichment media are liquid media. It permits the growth of certain pathogenic bacteria and simultaneously suppresses the growth of other bacteria. Examples of enrichment media are Selenite Cystine, Tetrathionate Brilliant Green and Rappaport Vassiliadis used for the separation of *Salmonella* spp. (Rall et al. 2005).
- Selective media are solid media (Prinzi and Rohde 2020) containing inhibiting substances to facilitate the growth of desired bacteria and inhibiting the growth of unwanted bacteria. Some examples of selective media include MacConkey agar (Prinzi and Rohde 2020).
- Differential or indicator media are used to differentiate one microorganism from another growing on the same one media by observing their growth pattern. Examples of differential media include MacConkey agar (Prinzi and Rohde 2020).
- Transport media are used when some delicate organisms are needed to be transported. They are used so that the viability of the delicate organisms is maintained during their transport. They only contain buffers and salt and lack all other essential substances such as carbon, nitrogen and organic growth factors. Example of transport media is Stuart's transport medium used for *Neisseria gonorrhoeae* (Ebright et al. 1982).

1.6 Microbial Nutrition

Microorganisms have different types of nutritional needs. Bacteria represent the range of nutritional variety. Bacteria use both complex organic and inorganic substances for their survival. Bacteria represent the boundary between these two types of nutrition (Lundgren 1989). The sources of energy for the microorganisms are carbohydrates, alcohols and amino acids. Most microorganisms are capable of metabolizing simple sugars such as glucose and also complex carbohydrates, such as starch or cellulose, or glycogen in muscle foods to obtain energy, while other microorganisms metabolize fats to obtain energy (Sandle 2015). Metabolism in microbes takes place in two ways—catabolism and anabolism catalysed by enzymes. Catabolism is a process of breaking down of large molecules sequentially in a stepwise manner with the release of energy which is trapped in the chemical bonds, and anabolism is the reverse process which uses up the energy from molecules for biosynthesis (Fani 2012). Also microorganisms obtain their energy by trapping of light energy and ultimately convert it into chemical energy. The kinds of nutrients and how they are assimilated and fed into the various metabolic pathways for energy production and utilization in microorganisms is the subject matter of this section.

1.6.1 Nutritional Classification of Prokaryotes

Microbes are grouped according to their requirements for energy from different sources of food (Chan 2003). Both the prokaryotes and the eukaryotes display exceptional nutritional mode.

1.6.1.1 Modes of Nutrition in Prokaryotes

Prokaryotes are diverse in their way to obtain resources and energy to synthesize organic compounds. The ones that utilize light energy are known as phototrophs, and the ones which use chemicals from the environment are known as chemotrophs. When prokaryotes utilize inorganic carbon source of energy, they are known as autotrophs, and others that utilize organic nutrient as carbon source are known as heterotrophs. A combination of the former two terms along with those used in describing carbon utilization results in the following nutritional types:

- *Chemoautotrophs*: microbes that utilize inorganic chemical substances such as H_2S, NH_3 and Fe^{2+} to obtain energy and carbon dioxide as the main source of carbon
- *Chemoheterotrophs*: microbes that use organic carbon as sources of energy
- *Photoautotrophs*: microbes that use light energy to synthesize organic compounds from CO_2
- *Photoheterotrophs*: microbes that use light energy and organic components as the main source of carbon

The chemoheterotrophs are very diverse and unique in the group—some have very specific and stringent requirements, while others are flexible in their requirements. For example, lactobacillus will grow well only when the medium contains certain other molecules such as amino acids, several B vitamins, ascorbic acid, glucose, acetate and oleate (Guirard and Snell 1964), whereas *Eschericia coli* is a chemoheterotroph which can grow on only sugars or amino acids or mixtures of both (Boncristiani et al. 2009).

Microorganisms are grouped according to hydrogen atoms or electron requirement.

Microorganisms that use reduced inorganic compounds as their electron source are called lithotrophs. And the ones that obtain electrons or hydrogen atoms organically are called organotrophs (Chan 2003). These groupings are further subdivided as follows:

- Photolithotrophic autotrophy
- Photoorganotrophic heterotrophy
- Chemolithotrophic autotrophy
- Chemoorganotrophic heterotrophy

1.6.2 Microorganisms Employ Metabolic Pathways to Metabolize Glucose and Other Biomolecules

- *Nitrogen metabolism*: Prokaryotes can metabolize nitrogen compounds. Nitrogen is an important biomolecules required for survival of the microbes. Hence they play an important role in the nitrogen cycle of the ecosystems (Bernhard 2010).
- *Oxygen metabolism*: Bacteria can be grouped on the basis of their requirement or sensitivity to oxygen (Coleman and Smith 2007).
  - Obligate aerobes obtain most of their energy by oxidative phosphorylation using O_2 as the terminal electron acceptor in the process.
  - A facultative anaerobe survives via anaerobic respiration. They can tolerate both the presence and absence of oxygen.
  - Obligate anaerobes survive without the requirement of molecular oxygen. They are highly sensitive to O_2 which leaves them unfit to grow in its presence.
  - Microaerophilic organisms best grow at lower oxygen level concentrations and their growth is inhibited when the concentration increases.
- *The evolution of glycolysis*: Since the evolution cells were able to obtain food from the organic molecules present in the environment. But everything reaches a threshold limit and becomes self-limiting; hence prokaryotes needed to resort to an alternative source and evolve a self-mechanism to generate energy and synthesize the biomolecules required for their survival and growth. The first prokaryotes, probably the chemotrophs which evolved 3.8 billion years ago, absorbed free organic compounds (including ATP) via abiotic metabolic activity. All cells use adenosine 5′-triphosphate (*ATP*) as their source of energy to carry on their cellular activities. However as ATP supplies were low, prokaryotes resorted to an alternate way to regenerate ATP from ADP, leading to evolution of glycolysis (Fani 2012).
- *The evolution of electron transport chains and chemiosmosis*: The process tells us how microbes can obtain energy by some other methods instead of from high-energy molecules. The chemiosmotic theory states that energy-generating membranes, such as cell membranes, mitochondrial membranes and chloroplast membranes, pump protons across the membrane that generates an electrochemical gradient of protons across the membrane. ATP is used as energy molecules to drive the pumps (Alberts 2002).
- *The evolution of photosynthesis*: With the evolution, free ATP and abiotically produced organic molecules were depleted, and therefore nature resorted to an alternative way where organisms could make their own organic molecules from inorganic resources. Hence, the development of photosynthesis allowed the cell to utilize energy from sunlight, instead of from other organic molecules for driving their metabolic requirements (Blankenship 2010).
- *The evolution of cellular respiration*: Cyanobacteria are the bacteria that can perform aerobic photosynthesis and respiration simultaneously in the same location, and some cyanobacterial species can also fix nitrogen (Vermaas 2001).

1.7 Growth in Microbes

Microbial growth is increase in population which is either the increase in cell number or the mass (Maier and Pepper 2015). Microbial growth is important parameter in microbiology for the examination of activities such as cell growth and biocatalytic functions. There are techniques available to observe cell growth by direct or indirect measurements. Certain factors are considered while calculating microbial growth, such as cell dry weight of the cell, optical density of the cell, turbidity, cell respiration, substrate utilization and product formation (Najafpour 2015). Microbial growth is achieved by cell division which leads to the growth of cell population. They are of two types:

- *Binary fission*—Bacteria divide by fission. The bacterial cell becomes double in size and replicates its genetic material (DNA) present in the chromosomes (Margolin 2014).
- *Budding*—Some bacteria reproduce by budding, forming a bubble-like structure that increases in size and detaches from the parent cell (Hirsch 1974).
- *Bacterial Growth Curve*: Measuring bacterial growth is an important characteristic in microbiology for its various applications in everyday life. It can be done in two ways—closed system or batch culture and continuous culture system (Maier and Pepper 2015). Batch culture is a culture where no nutrients are renewed or wastes removed. Hence the exponential phase is maintained till a limited period of time. Here in this culture, the bacteria grow in a particular pattern leading to growth curve composed of four distinct phases of growth: the lag phase, the exponential or log phase, the stationary phase and the death or decline phase. Growth curve can also tell about the generation time for an organism—the time for the population to double. The details of the growth curve having the different phases will be discussed later in this section.
- *Measuring Microbial Growth*: As mentioned microbial growth can be measured in two ways (O'Toole 1983):
  - *Indirect Measurements*: In this method the property of the mass of cells is measured.
    - *Turbidity*: It involves a colorimeter that measures the cloudiness or turbidity of the culture as cell numbers increase. Results are calculated by comparing the light absorbance with a standard graph plotted against known cell numbers.
    - *Metabolic Activity*: The metabolic activity is observed by calculating the rate of formation of metabolic products, such as gases or acids in the culture medium, and rate of utilization of a substrate such as oxygen or glucose.
    - *Dry Weight/Wet Weight*: Measuring the weight of cells both in dry or wet condition after centrifugation.
  - *Direct Measurements*—This method gives more exact measurements of numbers of microbes.

Direct Counts—It measures only bacterial cells. It measures both live and dead cells. It uses Coulter counter—electronic counter.

Plate Count—It is the indirect way of counting viable cell. When organisms are plated on a suitable medium, each viable cells grows and forms a colony which is counted as a colony forming unit (cfu).

Filtration—It is used for the measurement of very small quantities of bacteria. The pores in the filter do not allow microbial cells to escape. The filter retaining the cells is then placed on a medium and incubated and observed for viable cell growth.

Most Probable Number (MPN)—It is used to measure bacterial cells that are incapable of growing on solid medium.

1.7.1 Factors Affecting Microbial Growth

There are several factors which control the growth of microorganisms. The most important factors that affect microbial growth are the following: (1) "intrinsic factors" which include nutrient content, water activity, pH value and the presence of antimicrobial substances; (2) "extrinsic factors" which include temperature, composition of gases, relative humidity and pH; (3) "implicit factors" related to the microorganisms themselves, which include interactions between the microorganisms, their abilities to utilize different types of nutrient sources and tolerate stress, etc.; (4) "processing factors" which include heating, cooling drying; and (5) "the combined effects" which include interaction between the above stated factors that also affect the growth of microorganisms (Abo-Elenain 2017). Some of the factors are discussed as follows:

- *Intrinsic Factors*: Intrinsic factors are those that are characteristic of the food itself. They include the following:
  - *Nutrients*: Microorganisms require nutrients for its growth and maintenance of metabolic activities. These nutrients include water, energy source, nitrogen, vitamins and minerals (Mossel et al. 1995). Microorganisms primarily obtain carbon and nitrogen from biomolecules such as proteins, fats and carbohydrates. Some microorganisms search for it outside and absorb such food particles. Others may undergo biochemical reactions with surrounding elements such as carbon dioxide to obtain their needs, while others perform photosynthesis and obtain their sugar as a source of energy. On the other hand, microbes obtain nitrogen, required to synthesize proteins, through nitrogen cycle or from other organic matter (Hayatsu et al. 2008). The nutrient that microbes obtain and their growth are closely related. This relation between the nutrient concentration and microbial growth is expressed mathematically by Monod equation which is mathematically related to the Michaelis-Menten equation of enzyme kinetics. It states about the relation between microbial growth and rate-limiting enzyme reaction (Okpokwasili and Nweke 2006). Monod equation is designated by:

$$\mu = \mu_m S/S + K_s \quad (1.1)$$

where:

μ = specific growth rate
μ_m = maximum specific growth rate
S = concentration of limiting nutrient
K_s = saturation constant

- *pH*—Microorganisms, such as bacteria, are responsive to the hydrogen ion concentration. They have to maintain their pH above a significant point at or below which their protein denatures. Certain mechanisms are there that help bacteria to maintain intracellular pH. These mechanisms include homeostasis response, acid tolerance response and synthesis of acid shock proteins. Most bacteria grow best at neutral pH values (6.5–7.4); however some flourish in extreme acidic condition. Based on their property to tolerate different pH, bacteria are divided into the following categories (Keenleyside 2019):
  - *Acidophiles* or acid-loving—They grow at a pH of 1–6.8; examples are sulphur-oxidizing *Sulfolobus spp*. (Yellowstone National Park) (Brock et al. 1972), archaean genus *Ferroplasma spp.* (acid mine drainage) (Dopson et al. 2004) and *Lactobacillus spp.* (fermented milk) (Parker et al. 2018).
  - *Neutrophiles*—They grow at pH of 5.4–8.5; examples are *Escherichia coli*, *Staphylococci spp.* and *Salmonella* spp. (Keenleyside 2019).
  - *Alkaliphiles* or base loving—They grow at pH 7.0–11.5; examples are *Vibrio cholerae* (causes cholera) and the bright pink halophilic archaean *Natronobacterium*, found in the soda lakes of the African Rift Valley (Keenleyside 2019).
- *Redox potential*: A compound's redox potential, or more precisely, its reduction potential, relates to its tendency to receive electrons and hence be reduced (Prévost and Brillet-Viel 2014). The redox potential is measured in terms of millivolts. The redox potential is dependent on the pH of the substrate; redox potential is normally measured at pH 7.0 (Jay 2000). Examples of microorganisms whose growth is dependent on redox potential are aerobes, anaerobes, facultative aerobes and microaerophiles.
- *Water activity*: The water activity of food (a_w) is the measure of the amount of water "available" in food. Water availability (water activity a_w) is the vitality and functional activity of living systems (Stevenson et al. 2015). The water activity is expressed in terms of value ranging from 0 to 1. Pure water is expressed as 1, which signifies presence of all water, whereas a product with no water at all has a water activity of "0". Majority of microbes do not reproduce below 0.900 a_w (Bader et al. 1970; Manzoni et al. 2012; Moyano et al. 2013); except for the most extremophiles, cell division takes place at 0.61 a_w (Pitt 1975; Williams and Hallsworth 2009). The water activity of archaeal and bacterial cell at which their cell divides is between 1 and 0.755 (Anderson 1954; Grant 2004).

  - *Antimicrobial agents*: An antimicrobial agent is a natural or synthetic substance that either kills or inhibits the growth of microorganisms (Burnett-Boothroyd and McCarthy 2011). There are two types of antimicrobials: microbiostatic compounds that stop growth of microorganism and microbicidal compounds that kill microorganisms. There are a number of plant-based antimicrobial constituents which are microbicidal in nature (Jay 2000).
- *Extrinsic factor*: Environmental factors such as relative humidity, temperature, etc. determine the presence of competitor microbes that affect the microbial growth.
  - *Relative humidity*: Different types of microbes require varied amounts of water in the form of vapour for their activity. Microbes require relative humidity (RH) of 60% or more for their survival, whereas some can survive and multiply in >20% RH (Arfalk 2015). Temperature: Temperature and gas composition are the main extrinsic factors influencing microbial growth. Temperature has profound influence on growth and physiology. Temperature has influence on gene expression as well. Cells grown at refrigeration temperature grow differently than those at room temperature. Above and below optimal temperature, the growth rate decreases (Keenleyside 2019).
  - Effect of temperature on the growth of microorganisms:

    Psychrophiles—Psychrophilic are cold loving which inhabit cold environments such as deep sea, mountain and polar regions. They are further classified as the psychrotolerant or psychrotroph. Examples include *Moritella profunda* (D'Amico et al. 2006).

    Mesophiles—Mesophiles grow at moderate temperatures between 20 and 45 °C and at an optimum temperature in the range of 30–39 °C. They grow both in soil and water. Pathogenic microbes infecting animals and humans at temperature (37 °C) are mesophiles; Examples include *Streptococcus pyogenes* (Schiraldi and De Rosa 2014).

    Psychrotrophs grow at 0 °C. Psychrotrophs have a maximum temperature for growth above 20 °C and are widespread in nature. They are responsible for the spoilage of refrigerated food. Examples include the human pathogen *Listeria monocytogenes* (Gounot 1986; Farber and Peterkin 1991).

    Thermophile and hyperthermophile organisms grow at optimum temperatures ranging between 50 and 80 °C ("heat loving"). They are inhabitants of extreme habitats such as deep-sea hydrothermal vents, terrestrial hot springs and other extreme sites including volcanic sites, as well as decaying matters such as the compost (Panda et al. 2019). Examples include photosynthetic bacteria which generally grow up to 70–75 °C and include many cyanobacteria as well as green and purple sulphur bacteria (Holden 2009).
  - *Gaseous content*: Microbes are categorized in terms of their ability to use and tolerate oxygen. On the basis of their requirement to oxygen, microbes are categorized as follows (Singh et al. 2017):

    Obligate aerobes: They can tolerate and grow at full oxygen concentration (greater than 21%).

Microaerophiles: Microaerophiles can use oxygen when present at low levels in air (Rihane et al. 2020).

Facultative anaerobes: Facultative organisms can grow both in aerobic or anaerobic conditions. Example includes *Clostridium* and *Propionibacterium* (Stieglmeier et al. 2009).

Anaerobes lack respiratory systems and thus cannot utilize oxygen. There are two types of anaerobes:

Aerotolerant anaerobes can tolerate oxygen and grow in its presence even though they cannot use it.

Obligate anaerobes are killed by oxygen, for example, *Bacteroides fragilis* (Onderdonk and Garrett 2015).

Competitive microflora: The mutual interactions among different microflora greatly affect microbial growth (Boziaris and Parlapani 2017).

1.7.2 Microbial Growth Kinetics

Growth is a dynamic nature of living organisms. Growth kinetic studies tell about how all activities of microbial life relate to growth, survival, death, etc. (Panikov 1995). Most importantly kinetic model of cell growth predicts about product formation. It establishes a relationship between growth and environmental factors, especially the nutrients (Van Niel 1949). The growth of microorganisms is a highly composite and synchronized process expressed by rise in cell number or cell mass. Growth depends on certain factors such as the availability of nutrients, and other environmental factors. Different microbes have different growth patterns and each of them is unique. For example, bacteria divide by fission, yeast cells grow by budding, and viruses reproduce within a host (Wang and Levin 2009). The microorganisms can be grown in batch (Blaby et al. 2011), fed-batch (Stanbury et al. 2017), semi-continuous or continuous culture (Kuenen and Johnson 2009) systems in a bioreactor depending on nutrient supply (Table 1.1).

- *Batch Culture*: In batch culture the fermentation is performed in a closed system. Here the substrate and nutrients are added immediately after inoculation termed as zero time, and the incubation is carried out under optimal physiological conditions (pH, temperature, O_2 supply, agitation, etc.). Alkali or acid is added to maintain the pH and antifoam agent is used to avoid over-foaming. Batch culture is determined by certain growth patterns or phases (Maier and Pepper 2015):
  - *The Lag Phase*: The period immediately after inoculation is the lag phase. In this period the microorganisms try to adapt to their new environment such as available nutrients, pH, temperature, pressure, etc. This period is characterized by slight increase in cellular weight but no increase in cell number. Multiple

Table 1.1 List of some bioreactors and their application

Serial no	Bioreactor	Function	Reference
1.	Super-high-rate anaerobic bioreactor	Wastewater treatment	Abdelgadir et al. (2014)
2.	Biologically active carbon reactor	Removal of perchlorate and nitrate from contaminated water	Li et al. (2010)
3.	Continuously stirred tank bioreactor	Wastewater treatment	Gargouri et al. (2011)
4.	Air lift	Decolorizing of textile dye using fungi	Mahmood et al. (2015)
5.	Slurry phase	Bioremediation of soil	Quintero et al. (2007)
6.	Fluidized bed	Pharmaceutical industry	Gros et al. (2014)
7.	Packed bed	Bioremediation of chemicals from industries	Liu et al. (2017)
8.	Biotrickling filter	Wastewater treatment	Olivieri et al. (2012)
9.	Woodchip bioreactors	Removal of nitrate from agricultural wastewater	Jéglot et al. (2021)
10.	Membrane bioreactor	Saline wastewater treatment	Tan et al. (2019)

lag phases might occur if the media are supplemented with multiple glucose nutrient, and such type of growth is referred to as diauxic growth.
  - *The Log Phase*: The lag phase is followed by log phase in which there are an exponential growth of cell mass and increase in cell number and at the same time nutrients are exhausted leading to deceleration phase.
  - *The Deceleration Phase*: This phase is characterized by accumulation of toxic compounds. This period is short.
  - *The Stationary Phase*: As the nutrient in the growth medium gets exhausted, and the metabolic end products that are formed in the process inhibit the growth, the cells enter the stationary phase. The microbial growth slows down gradually. The biomass produced remains constant during stationary phase and it is in this phase the secondary product or the antibiotic is produced.
  - *The Death Phase*: This phase is associated with termination of metabolic activity and decrease of energy reserves. The cells die at an exponential rate in this phase. This phase is of huge application in the commercial and industrial fermentations as the cells are harvested in this stage after the cells halt their metabolic activities finally.
- *Fed-Batch Culture*: Fed-batch culture is an improvised version of batch fermentation wherein the substrate is added in instalment at different stages during the ongoing fermentation. The periodical addition of substrate increases the time period for log and stationary phases which results in an increased biomass leading to production of and also increases in production of metabolites such as antibiotics.
- *Continuous Culture*: Continuous culture cultivates microorganisms at submaximal growth rates at different growth parameters in such a way that the culture

conditions remain constant (in "steady state") over long periods of time. Continuous culture principles were first applied in the labs of Herbert, Monod and Novick. Continuous fermentation is carried out in an open system. The steady state is maintained with simultaneous removal and replacement of culture medium to keep the working volume constant in a bioreactor. A bioreactor is a vessel in which a biological reaction takes place. A bioreactor includes all the parameters required for the growth of microorganisms. The bioreactor is a place where an optimum external environment is maintained to support a high yield biological process. A bioreactor is available both for plant metabolites and microbial metabolites.

1.7.2.1 Measurement of Microbial Growth Kinetics

Growth kinetics study tells about the direct proportionality between growth and the concentration of cell (Sakthiselvan et al. 2019). The concentration of cell is measured by both direct and indirect method as already mentioned in previous section. One direct method is to use the spectrophotometer to measure the absorbance of cell suspensions. Using this technique the absorbance is plotted as a standard curve versus cell concentration and thus the cell concentration of an unknown sample determined. A counting chamber can be used to perform direct microscopic counts of cells. For this, electronic counting chambers can be employed. Indirect viable cell counts can be done with plate counts by diluting cultures on nutrient medium. Turbidity is another way of measurement for counting large numbers of bacterial cells in clear liquid media and broths—but cannot count cell densities less than 10^7 cells/mL. Measuring biochemical parameters such as O_2 uptake, CO_2 production and ATP production is another method to count cell culture. Bacterial growth rates or the generation time is measured during the exponential growth, under the influence of standard nutritional conditions such as culture medium, temperature, pH, etc. Bacterial cells multiply exponentially by binary fission and the increase is a geometric progression. The generation time is the time interval required for cells' population to divide and designated by the following equation (Todar 2020):

$$G = t/n \tag{1.2}$$

where:

G is generation time
n is number of generations
t is time in min/h

The equation for growth by binary fission is:

$$b = B \times 2^n \tag{1.3}$$

where:

b is number of bacteria at the end of a time interval
B is number of bacteria at the beginning of a time interval
n is the number of generations (number of times the population doubles in the time interval)
$\log b = \log B + n\log 2\ n$
$n = (\log b - \log B)/\log 2$
$n = (\log b - \log B)/0.301$
$n = 3.3\ \log b/B$

$$G = t/3.3 \log b/B \tag{1.4}$$

Growth kinetics can be classified based on relationship between synthesis of product and generation of energy in the cell such as:

- Growth associated: Product formation is related directly to growth of cells.
- Non-growth associated: The products formed are the end product of the metabolic activity of the cell. The byproducts formed are called secondary metabolites. Product formed is not linked to growth rate but it is a function of cell concentration.
- Mixed-growth associated: The formation of product depends on both growth rate and cell concentration.

Product kinetics: Microbial growth kinetics is the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (s). It is an important tool in microbiology. Of all the different kinetic equation proposed, Monod equation is most accepted. The model specifies that the microbial specific growth rate μ depends on the growth-limiting substrate (S) (Manikandan et al. 2008; Song et al. 2010) such that:

$$\mathrm{d}x/\mathrm{d}t = \mu x \tag{1.5}$$

$$\mu = \mu_{\max} S/(K_s + S) \tag{1.6}$$

where μ is specific growth rate (day^{-1}), $\mu_{\max}$ is maximum specific growth rate (day^{-1}), K_s is half-saturation constant (mg/g), x is microbial cells measured as glucosamine concentration (mg/g), and S and t are initial substrate concentrations (mg/g) and time (day), respectively.

Conclusion: Kinetic model studies have wide application in the evaluation of the product formation kinetics. The mathematical model helps in the data analysis and provides a solution to difficulties experienced in fermentation. It also helps in the batch process performance.

1.8 Maintenance and Preservation of Microbial Cultures

Microorganisms are important both commercially and ecologically (Hayat et al. 2010; Senni et al. 2011). Other than that they are considered as the support system of biotech industry (Stahl and Wagner 2006; Vibha and Neelam 2012) and are also natural source of novel therapeutics and biomolecules used in pharmaceutical industry (Zaidi et al. 2009; Hayat et al. 2010). Therefore, proper maintenance and preservation of microbial cultures is a prerequisite to ensure long-term availability and viability of microbes without losing their morphological, physiological and genetic traits (Ward et al. 2001). Agar slant culture, stab culture, saline suspension, glycerol and DMSO preservation, drying on silica gel, drying on soil, sterile water, lyophilization, cryopreservation, etc. are the laboratory techniques used for preservation of microorganism. However, the choice of method to be used depends on type of microorganism to be used and purpose of and duration of preservation. In this regard, culture collections (CCs) and microbial biological resource centres (mBRCs) play an important role in the preservation and circulation of biological resources. Both are ex situ repositories for biodiversity and provide collection of useful microorganisms and related information, for research (Caktu and Turkoglu 2011; Stackebrandt et al. 2014). Preservation of microbes is a very old process (Nakasone et al. 2004). Different types of methods have been regularly employed for the preservation of microorganisms which include repeated subculturing, on agar beads (Winters and Winn 2010), oil overlay of slant-grown cultures (Nakasone et al. 2004), use of silica gel (Liao and Shollenberger 2003), cryopreservation (Gorman and Adley 2004) and lyophilization (Berner and Viernstein 2006). Here in this section, we shall discuss in brief the techniques used in preservation of microbes for long-term and short-term storages.

- *Continuous culture*: In this method cultures are grown in agar for short-term storage and are stored at 4–21 °C to delay the time period required for subculturing. Fresh cultures of the strain can be maintained from the previous stock culture. This method is usually done in an agar slant or agar stab media in a test tube. Medium used is nutrient agar or potato dextrose agar. In this medium the cultures remain viable for weeks to several months (Kumar et al. 2013).
- *Drying method*: It is the most useful method for storing produced spores. Silica gels, glass beads and soil are the common media for drying. For example, fungi have been stored in silica beads in this way for years (Kumar et al. 2013).
- *Refrigeration*: Using this method the microbial cultures in agar slant, stab media or Petri plate are stored at 4 °C. The cultures are properly sealed (Kumar et al. 2013).
- *Mineral oil/liquid paraffin storage*: Fresh grown cultures in slants can be covered in mineral oil or liquid paraffin to preserve the culture for longer period of time and to cut off the access to oxygen to slow down the metabolic rate and prevent the cultures from drying. The most commonly used oil is paraffin oil or Vaseline with a thickness of 1–2 mm (Kumar et al. 2013).

- *Freezing*: This technique is the most preferred technique to keep the cells viable at very low temperatures. Freezers used for this are of three types (Kumar et al. 2013).
- *Freezing bacteria using glycerol*: Though freezing has its advantage, there is certain drawback about it. The ice crystals formed might damage the viability of the culture by dehydration caused by increase in local concentration of soil. To lessen the effect of freezing, cultures can be preserved using 15% glycerol. The microfuge tubes with screw cap and sterile glycerol are used. The glycerol is diluted to 30%, and equal amount of glycerol and broth are mixed along with the culture and then frozen (Kumar et al. 2013).
- *Freeze-drying or lyophilization*: Lyophilization involves removal of water from the sample which serves a purpose for enzymatic reaction. It freezes sample with the aid of a lyoprotectant (usually a sucrose) and pulling out of water using a vacuum helps in the preservation of cultures (Prakash et al. 2013). Lyophilization preserves many bacteria, yeast and sporulating fungi, but does not preserve non-sporulating fungi.
- *Cryopreservation:* Cryopreservation uses preservation of biological materials at cryogenic temperatures, at −80 °C (dry ice) or at −196 °C (liquid nitrogen). Low temperature acts as a protective shield for proteins and DNA from denaturation and damage and slows the water movement in the cell. As a result, biochemical and physiological activities of the cells are kept halted, and cells are preserved for long periods of time (Prakash et al. 2013).

1.9 Strain Improvement

Strain improvement is the technology of genetically changing microbial strains to improve their potential and metabolic activity. Genetically improved strain has several application in numerous biotechnology applications, fermentation techniques and assay. Wild strains are not capable of producing high yield metabolites; however the yield can be increased by optimizing the fermentation process. Metabolite production is genetically determined; therefore new genetic strains have to be developed for increased product formation in a cost-effective way. Successful development of improved strains requires a proper knowledge of physiology and metabolic activity (Parekh et al. 2000). Microorganisms produce new genetic characters ("genotypes") by two processes: mutation and genetic recombination. Mutations are of two types: spontaneous mutation or induced mutation. The benefits of mutation are of application to humans. This has been a breakthrough in the fermentation industry. The main development of microbial genetics began in the late nineteenth century with the onset of penicillin production. The benefits of mutation led to a growing interest to genetically engineer microorganisms. Thus the collaborative "strain-selection" experimental programme was started among researchers at the US Department of Agriculture laboratories in Peoria, the Carnegie Institution, Stanford University and the University of Wisconsin, followed by the extensive worldwide strain improvement programme

in various industrial laboratories throughout the world to develop genetically improved strain (Adrio and Demain 2006).

1.9.1 Applications of Mutation

Mutation has a great role in improving the yield of microbiological product (Vinci and Byng 1999). The spontaneous mutations are of low frequency, not suitable for industrial purposes. Therefore mutations may be induced in the laboratory by mutagenic agents such as ultraviolet light and various chemicals (nitrosoguanidine (NTG), 4-nitroquinoline-1-oxide, methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS) and hydroxylamine (HA)). Site-directed mutagenesis is also important for strain improvement. The strain that survives mutation is plated out, selected randomly for growth in flasks for future prospects (Simpson and Caten 1979). Selection of mutants can be done in two ways (Schwab 1988):

- *Random screening*: The mutated strain is selected for their ability to produce the desired product with a model fermentation unit. The strain with the maximum yield is selected.
- *Selective isolation of mutants*: There are several ways such as:
  - Isolation of antibiotic resistant strain: Both the wild and the mutant are grown in a media containing antibiotic. The wild strain dies out and the mutant strain with the antibiotic strain survives.
  - Isolation of antimetabolite strain: Antimetabolite having structural similarity to metabolite may inhibit the metabolic pathway and kill the cell. Mutant strain having resistant gene to the antimetabolite survives and can be selected for industrial purpose.
  - Isolation of auxotrophic strain: An auxotrophic may be characterized by detecting the defect in one of the biochemical pathways.

1.9.2 Recombination

The strain improvement can be done by exchanging of genetic information between two strains, by a process called genetic recombination. The recombination can be done by transformation, transduction, conjugation and protoplast fusion. Protoplast fusion is in much use.

- *Protoplast fusion*: Since sexual reproduction does not exist in prokaryotes, therefore, genetic recombination in microorganisms is done by parasexual mechanisms which is not of much efficiency. Protoplast fusion techniques are of great advantage to be applied to newly isolated, not fully characterized, strains (Ferenczy 1981).

- *Recombinant DNA*: Recombinant DNA technology (genetic engineering) is the combination of all those newly developed methods that allows for the isolation, characterization, manipulation, expression of specific genes, etc. rDNA technology can transfer specific genes between organisms. Genetic engineering has been instrumental instrument in the production of mammalian proteins by microorganisms; examples include human insulin, interferon blood proteins, peptides of the neurosystem and also proteins used as vaccines which are presently under development (Riggs et al. 1984). Organisms such as *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* are used as hosts (Schwab 1988). Cloning systems have now been developed for use in genetically engineered strains for commercial purpose.
- *rDNA technology*: rDNA techniques for strain improvement are used for the controlled expression of specific genes. Such genes are obtained either from homologous or from heterologous systems with the aid of cloning using shuttle vectors. Three points have to be considered to have an effective rDNA technology for its application in microbial system (Schwab 1988):
  - DNA transfer systems
  - Vectors' availability with selection systems
  - Efficient functional control elements for gene expression
- *DNA transfer systems*: DNA transfer into living cells is an important requirement. The following strategies may be applied to achieve so (Schwab 1988):
  - Transformation
  - In vitro packaging of DNA into phage heads and transferring to host cells via phage infection
  - Microinjection
  - Conjugative plasmid transfer from an intermediate host

1.10 Microbe-Human Interdependence

It may appear to us that we humans are a single living creature embodying flesh and blood, but that's not the entire truth. We are in fact full of microscopic organisms that are found inside and outside of the body. Such microscopic organisms which are known as microbiome have advantages on our physiological and mental health (Johnson and Versalovic 2012). Today, researchers have realized the need for a more holistic approach towards the microbial world around us: Microbes and their hosts (including humans) are interdependent for survival. All organisms are not harmful; some are beneficial too. Scientists believe that about half of all human DNA genetic material originated from viruses that infected our ancestors' egg and sperm cells. In fact, our bodies contain at least ten times more bacterial cells than human ones (Sender et al. 2016). Microbes present in the human gastrointestinal tract alone comprise at least ten trillion organisms, representing more than 1000 species, which prevent colonization of pathogens in the gut (Drexler 2010). Due to limitations in

technology, the complex and dynamic nature of our microbiota was not fully recognized. Recent endeavours such as the Human Microbiome Project (HMP) (Peterson et al. 2009), hosted by the US National Institutes of Health, and the Metagenomics of the Human Intestinal Tract (MetaHIT) project (Ehrlich and MetaHIT Consortium 2011), through the European Commission, have initiated extensive research aimed at surveying and analysis of the collection of microbial genes and genomes collectively termed the microbiome. The efforts of the HMP have produced >70 million 16S ribosomal gene sequences characterizing the microbial communities across 15 body sites and >3.5 tera-base pairs (Tbp) of whole-genome shotgun metagenomic data encoding >60 million predicted genes (Methé et al. 2012; The Human Microbiome Project Consortium 2012).

1.10.1 Health and Disease

Microorganisms have profound impact on human health via different mechanisms. One such component linked to human health is pathogen-associated molecular patterns (PAMPs). PAMPs are molecules such as endotoxin and lipopolysaccharide (a component of bacterial cell membranes), flagellin (from bacteria) and (1–3)-β-D-glucans (also referred to as triple helical glucan, fungi wall membrane component). These molecules are found in microbes (bacteria or fungi) which affect human innate immune system and epithelial cells in the respiratory tract (Lambrecht and Hammad 2013, 2014). For example, many indoor fungi produce such metabolites that can induce respiratory allergic response (Kuhn and Ghannoum 2003).

The relationship between humans and microbes is profound. Both are dependent on each other through benefits and loss (Hess et al. 2011). For example, connection has been found between the resident gastrointestinal microbiota and atherosclerosis. A metabolic pathway was diagnosed that involves microbial metabolism of dietary phosphatidylcholine, resulting in the synthesis of the metabolite trimethylamine N-oxide (TMAO), a biomarker for cardiovascular disease (Wang et al. 2011). A recent study described the generation of the microbiota among individuals with a genetic link for celiac disease (Sellitto et al. 2012). Interest in proper understanding of the human microbiota consortia in relation to health and disease is fundamental for the development of personalized medicine.

1.10.2 Gut Microbiome

The human gastrointestinal (GI) tract contains complex and dynamic population of microorganisms, the gut microbiota, which has a great impact on the host in health and disease. The microorganisms inhabit the gut during infancy, and factors like diet are responsible for the establishment of gut microbiota within a lifetime (Thursby and Juge 2017). The collection of different types of microbes such as bacteria, archaea and eukaryote colonizing the GI tract is termed as the "gut microbiota". They have co-evolved with the host over the past years to form a mutually beneficial

relationship (Bäckhed et al. 2005). It is estimated that 10^{14} number of microorganisms inhabit the GI tract (Bäckhed et al. 2005; Gill et al. 2006). However, a recent revision of the survey has reported that the ratio of human-bacterial cells is actually closer to 1:1 (Sender et al. 2016). Growing interest towards the human gut microbiome has increased in recent dates due to the rapidly advanced technology such as sequence-based screening and human gnotobiotic model in analysing the dynamism of commensal microbiota. As mentioned gut microbiome plays a pivotal role in maintaining the immune and metabolic homeostasis of the body. Therefore gut microbial imbalance (dysbiosis) may lead to faulty host machineries, thereby contributing to pathogenesis and associated broad spectrum of illness. Some of the most noteworthy diseases to be mentioned which are associated with gut microbiome dysbiosis include *Clostridium difficile* infection (infectious disease), inflammatory bowel disease (intestinal immune-mediated disease), celiac disease (multisystemic autoimmune disorder), obesity (metabolic disease), colorectal cancer and autism spectrum disorder (neuropsychiatric disorder). Analysis of this mutual relationship of human and microbe has great potential in future research in medical science. Novel therapies derived from microbiome studies such as faecal microbiota transplantation, probiotic and prebiotics have been implemented to correct the dysbiosis, thus revealing a new scientific breakthrough towards disease treatment (Kho and Lal 2018).

1.10.3 Emerging and Re-emerging Diseases

Emerging diseases are infectious disease whose frequency of occurrence in humans has increased within the recent past or threatens to augment in the near future. They may be the outbreaks of earlier unknown diseases that are rapidly increasing in occurrence or geographic area in the last two decades. These may include new or old diseases with new features. These new features may include the introduction of a disease to a new area or a new vulnerable population, with new clinical features, including resistance to available drug (Dikid et al. 2013). Many risk factors add to the emergency and rapid spread of epidemics in the region including severe and long-lasting humanitarian emergencies, resulting in weak health systems, increased population mobility, speedy urbanization, sudden climate change, weak surveillance and less laboratory diagnostic capacity and increased human-animal interaction (Buliva et al. 2017). Emerging diseases include lower respiratory tract infections, diarrheal diseases, HIV/AIDS, malaria and tuberculosis (TB), Middle East respiratory syndrome, extensively drug-resistant tuberculosis (XDR TB) and Zika virus (Morse 1995; Vos et al. 2015). Re-emerging diseases are the ones that make reappearance after they have been on a major fading phase. Re-emergence happens when there is a violation of protocol in public health measures for diseases that were contained. They can also happen if mutant strains of known disease-causing organisms emerge. Also human behaviour may be one of the reasons for re-emergence. For example, overuse of antibiotics has led to emergence of mutant strains that are resistant to existing treatment. It is reported that about 30 new

infectious disease-causing agents have been detected worldwide in the last three decades and most of them are zoonotic in origin, causing the emerging and re-emerging of infections leading to epidemics and pandemics. Besides health issues, emerging infections also affect the economic development. As such in the recent past, the emergence of SARS caused by coronavirus (Dikid et al. 2013) and again the re-emerging of the novel coronavirus infection in the form of Covid-19 have devastated the global economy.

1.10.4 Epidemic and Pandemic Diseases and Microbes

Infectious diseases leading to epidemics, pandemic and endemic have caused devastation to humanity throughout its existence, often changing the course of history. All these three words epidemics, pandemic and endemic are from Greek literature, and the one they have in common is demic (pertaining to people or population) (Bharat Biotech 2020).

- *The term epidemic* is used when an infectious disease affects a substantial number of people in a given city, region or country for a fixed period of time.
- *Pandemic* is when a particular outbreak extends beyond national borders, across a continent or even around the globe. It causes a deadly illness that is transmitted easily from one person to another, which spreads across a wide geographic area, affecting and even killing many people. In history, there have been a number of devastating pandemics. And the most impactful pandemic which till today has wrapped the globe is the coronavirus pandemic of 2020. On March 11, 2020, coronavirus was officially declared a pandemic by the World Health Organization.
- *An endemic* is the persistence of an infectious disease in a particular geographic region which affects a large part of the population. They remain at a stable state, but do not vanish from the population. For example, chickenpox is categorized as an endemic.
- *The term Outbreak* is used when the number of cases of a disease exceeds the normal expectation for the region or season.

The emerging infections may offer a real challenge for the entire globe. A strategic response must be approached towards the problem at the core level. A well-planned international strategy on fighting infectious diseases addressing the issues of emerging and re-emerging diseases should be taken up by both governmental and non-governmental agencies.

1.11 Benefits of Microbial Activity in Food and Industry

Nature utilizes microorganisms for fermentation, and since time immemorial mankind has used yeasts, moulds and bacteria to make food products such as bread, beer, wine, vinegar, yoghurt and cheese, as well as fermented fish, meat and vegetables without the prior knowledge of microbes being involved in the process. Fermentation is a traditional method for food transformation and preservation. This method allows to retain the nutritional and organoleptic qualities (relating to the senses: taste, sight, smell, touch) of food. The first discovery that microorganisms were involved in food production processes was made in the early years of 1800; yeast was found to have a role in alcoholic fermentation in the formation of beer and vinegar by French chemist and biologist Louis Pasteur (Pai 2003). However, it was during the World War I that the technological microbiology gained popularity. This started with the demand for glycerol for the manufacture of explosives during World War I (Wang et al. 2001) and the large-scale production of penicillin, discovered by Fleming, in the 1940s (Neushul 1993). The end of World War II witnessed the expansion of the American economy known as the Golden Age of Capitalism (Marglin and Schor 2000), and the growing interest in the knowledge of microbial genetics (Susman 1970; Bagdasarian 1982). This triggered the appearance of microorganism-based industrial processes, making way for modern technological microbiology. However, technological microbiology found legal validation in the 1980s, following a decision made by the US Supreme Court to patent *Pseudomonas putida* variant, by Ananda Chakrabarty, that it is efficient in naturally digesting compounds found in crude oil spills (Robinson and Medlock 2005). This patent was a revolution in biotechnology that resulted in the thousands of other patents that made way for establishing hundreds of bioengineering and food plants (Holloway 2015). Later with the progression of knowledge on genetic manipulation, the modification of *Escherichia coli* by genetic engineering led to the production of artificial insulin, which was the first product obtained and a breakthrough from recombinant DNA technology (Walsh 2012), approved by the US Food and Drug Administration in 1982 (Johnson 1983). Eventually applied microbiology became a science essentially applied to several branches of production, including food, chemical, agricultural and pharmacological. Here in this section, we present some application of microbiological fermentation in food industry.

1.11.1 Application

- *Production of organic acids*
  - Citric acid is produced by fermentation with *Aspergillus niger* in large corrosion fermenter using stirrer. The aid of yeast such as *Candida albicans* has also been useful in the process (Roehr 1996).
  - Lactic acid another important organic acid is produced by *Lactobacillus delbrueckii* (Rabbani Khorasgani and Shafiei 2017), *Lactobacillus casei* (Panesar et al. 2010) and *Lactobacillus acidophilus* (Bull et al. 2013).

  - *Gluconic acid*: It is produced by fermentation of *Aspergillus niger* (Shindia et al. 2006).
- *Production of industrial enzymes*: Enzymes have been used in food industry for various reasons such as making of bread, fermentation of juice and malt and clotting of milk to make cheese. Rennet a complex set of enzymes occupies 25% of worlds' market. It is produced in any mammalian (calf) stomach and is often used in the production of cheese. The active enzyme present in rennet is called chymosin or rennin. Glocoamylase (20%) and then glucose isomerase (5%) are high in demand for enzyme. Also genetically modified microorganisms such as *Eschericia coli* containing calf rennet were developed. *Bacillus spp*. such as *Bacillus acidopullulyticus* produces pullulanase; alpha-amylase is produced by *Bacillus amyloliquefaciens*, glucose isomerase by *Bacillus coagulans*, etc. (Pai 2003).
- *Production of amino acids*: Amino acids are an important component in food industry. Amino acids such as α-glutamate are used as flavour enhancer, glycine as sweetener, lysine and methionine as food and feed additive and phenylalanine and aspartame as a low calorie sweetener. All these are prepared either chemically or enzymatically. Microorganisms such as *Corynebacterium glutamicum* used for preparation of glutamate, lysine, threonine, etc. are used to produce these amino acids. Some genetically modified organisms such as *Escherichia coli*, *Serratia* spp., *Bacillus* spp., *Hansenula* spp., *Candida* spp., and Saccharomyces spp. are used in amino acid production (Pai 2003).
- *Effects of lactic acid fermentation on the nutritional aspects of food*: Lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, etc.) have been used to enhance flavour, texture, preservation and nutritive value of dairy products and other foods. Some fermented dairy products are also used as probiotics (Rezac et al. 2018). The quality of a food is dependent on nutrient content as well as the digestibility, which may be improved by the process of fermentation (Nkhata et al. 2018). Fermentation also has great impact on nutritional diseases (Hill et al. 2017). Fermentation of food contributes with the bioavailability of vitamins such as niacin, thiamine, folic acid or riboflavin (Melini et al. 2019). Iron absorption of food is enhanced by breaking it into inorganic iron with vitamin C (Nkhata et al. 2018). Food fermentation also helps in uptake of the mineral and trace elements by reducing the non-digestible content in plants such as glucuronic and polygalacturonic acids, cellulose and hemicelluloses (Gupta et al. 2015). It also reduces blood cholesterol by inhibiting cholesterol synthesis in the liver and also inhibits dietary cholesterol absorption in the intestine (Jesch and Carr 2017). Lactic acid fermentation product also provides relief to infections such as diarrhoea and salmonellosis (Minh 2014).
- *Alcohol production using improved cultures*: Yeast is used for beer brewing. Strains such as *Saccharomyces cerevisiae* and *Saccharomyces uvarum* have been commonly used (Pai 2003).
- *Miscellaneous application*: *Candida utilis* has been used in industry to produce single-cell protein to be used as food and fodder, waste treatment and production of flavour enhancer (Pai 2003).

1.12 Conclusion

- The introduction on how microbes impact our lives and are involved both in good health and sickness through their benefit and harm is elaborated. This chapter gave a clear insight into isolation, mass cultivation, growth kinetics and preservation strategies for the industrial application of microbes. The types of microbes based on their ability to grow in certain conditions were explained. Also, strain improvement strategies were discussed with their industrial implications along with an overview on how microbes can be useful for the generation of novel products that have extensive applications in the biotechnology industry. This interactive and engaging chapter will be useful in the fields across the biological, environmental and health sciences, and for the interested reader wanting to explore more about these ubiquitous microbes.

Conflicts of Interest The authors declare no conflicts of interest.

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Bacteria and Their Industrial Importance

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Abstract

Bacteria have been of immense importance to man and the environment. Apart from their role in geochemical cycling and eco-restoration of the environment, bacteria are widely used in industries to synthesize products beneficial to humans. The ever-increasing utility of bacteria in industrial applications has impelled the expansion of the microbial industries into more varied and valuable horizons. Microbial cells function as mini biofactories and synthesize novel, economically important products like enzymes, organic acids, vitamins, antibiotics, antibodies, hormones, carotenoids, steroids, alkaloids, alcoholic beverages, interferons, and vaccines. Apart from the synthesis of these products, bacteria are also involved in wastewater treatment, bioremediation, biomineralization, biorecovery of precious metals and metalloids, biocontrol agents, production of prebiotics and probiotics, biogas, and eco-friendly nanomaterial production. In this chapter, we present a summary of the wide range of industrial applications of bacteria ranging from beneficial products to rehabilitation of the environment.

Keywords

Antibiotics · Bacteria · Biofactory · Biorecovery · Bioinsecticides · Enzyme · Industry

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
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2.1 Introduction

Microbes are ubiquitous and have contributed immensely for the benefit of humankind for centuries. The diversity of microbes also implies that their enzymatic content and metabolic pathways would be varied and functionally more essential (Demain and Adrio 2008). Traditional ways of preserving milk, vegetables, and yielding products such as bread, beer, wine, distilled spirits, vinegar, cheese, pickles, and other fermented materials have existed for thousands of years. The earliest evidence of alcoholic beverages dates back to 7000 BCE in China where jars containing remnants of rice wine were discovered. Although fermentation of milk products has been occurring since 10,000 BCE, it was only in 1857 that Louis Pasteur observed that lactic acid fermentation takes place due to microbes (Mulligan and Gibbs 1993). Modern industrial microbiology began in the early twentieth century, which featured the first large-scale fermentations devoted to the manufacture of solvents, organic acids, vitamins, enzymes, and other products. The success of large-scale penicillin manufacture contributed to significant economic growth, following which other value-added products like enzymes and secondary metabolites, e.g., steroids, hormones, and production of antibiotics like penicillin and streptomycin, came into the forefront of industrial microbiology (Demain and Adrio 2008). Other major products of growing market relevance include amino acids, organic acids, and enzymes of new applications (Buchholz and Collins 2013). The process of production of metabolites by microbes has been utilized in biotechnological industries for the mass manufacture of vaccines, antibiotics, and enzymes. Microbial metabolites are of two types: primary and secondary; primary metabolites are synthesized by microbes during active growth and consist of products like amino acids, proteins, carbohydrates, ethanol, acetone, and others (Kumar et al. 2018). Secondary metabolites, on the other hand, are produced after the active growth phase of the microbes and do not directly influence the microbial growth but instead function as defense mechanisms against other microbes, plants, insects, or animals. Sometimes, secondary metabolites act as agents that aid in the symbiosis between the microbe and other living organisms like plants, nematodes, insects, and animals (Demain and Fang 2000).

The wonders of biotechnology are immense and have revamped almost every aspect of our lives. The first-ever large-scale production of a microbial metabolite was the mass production of citric acid by Pfizer in 1929, by growing *Aspergillus niger* in a sugar-rich medium at a pH of around 2.5–3.5 (Swain et al. 2011). Subsequently, in 1941, the war-stricken nations of England and the United States of America recognized the need for large-scale manufacture of antibiotics and approached Pfizer for the commercial production of penicillin where deep-tank fermentation processes were utilized to produce penicillin from mold (Lombardino 2000). Bioprocessing technology involves the use of biological science in industrial processes like large-scale fermentation where cells are grown for the production of various beneficial products (Soderberg 2014). This chapter explains the process of product formation from bacteria used in industries highlighting the advantages and

applications of the numerous products obtained from microbial fermentation and bioconversion with an emphasis on bacteria and their utility.

2.2 Fermentation: Stages and Product Formation Process

There are two stages in industrial fermentation processes comprising the upstream and downstream process (USP and DSP, respectively).

2.2.1 Upstream Processing (USP)

The USP is the initial step of the fermentation process, which involves the selection of inoculum strain, media formulation to favor the growth of inoculum/starter culture in bioreactors or fermenters. The media are formulated keeping in mind the inoculum growth requirements as well as the targeted product to be obtained. In order to obtain the desired product, the improvement of bacterial or other microbial strains with the desired phenotypes has been the basis of most industrial fermentation processes. Even today, much of the emphasis placed on improving microbial strains is the result of the diversity of the metabolites produced by microorganisms that have found novel applications in the food, chemical, agricultural, healthcare, and pharmaceutical industries (Han and Parekh 2005). The process of obtaining improved strains involves three steps:

- Mutation of a parent strain to introduce new genetic alteration in the genome.
- Random screening and assessment of mutagenesis survivors in small-scale fermentation vessels.
- Quantification of the metabolite and identification of potentially improved strains.

In industrial settings, this medium becomes the starting point for further optimization. The primary drive here is to increase product titer and to improve the economy of a fermentation process. A secondary drive includes the development of a simplified metabolite-purification process (Han and Parekh 2005). The advent of recombinant DNA has brought about many changes to industrial microbiology. New expression systems have been developed, biosynthetic pathways have been modified by metabolic engineering to give new metabolites, and directed evolution has provided enzymes with improved catalytic activity and stability (Demain and Adrio 2008).

The fermentation process is carried out in batch-wise or continuous cultures. Batch or fed-batch processing is more commonly employed in industrial fermentations and is initiated by the inoculation of starter culture into a sterile medium. The fermentation duration or batch time may vary from a few days to even several weeks and months. Fed-batch fermentation differs from batch fermentation in a single step that includes the re-addition of sterile medium either periodically or continuously, and at the end of the batch time, the product is harvested

(Ingledew and Lin 2011). In the case of continuous cultures, the starter culture is inoculated into the sterile medium, and after the microbial density attains an optimal level, the sterile medium is pumped into the fermenter or bioreactor with simultaneous pumping out or harvesting of the products (Lindskog 2018).

2.2.2 Downstream Processing (DSP)

The products harvested after fermentation are usually present along with other biomolecules and impurities. DSP that involves concentration and purification of the product is a crucial step of the entire fermentation process. Since products like vitamins and enzymes are produced in low concentrations, it is a requisite measure to remove impurities as well as any other biomolecules that might inhibit the optimal functioning of the product. Separation techniques like filtration and centrifugation help to remove solid cells from the medium, and retention of either (cells or medium) depends on whether the product is synthesized intracellularly or secreted extracellularly. In the case of intracellular production, the cells are ruptured by any of the physical (grinding, milling), chemical (detergents, surfactants, alkalis), or biological (antibiotics) techniques (Clarke 2013). The product recovery step involves several separation and purification techniques like precipitation, liquid extraction, chromatography, membrane separation, and electrophoresis. The nature of the product determines the steps of DSP to be undertaken in such a way that it maximizes efficiency while also reducing the production cost (Łojewska et al. 2016). Figure 2.1 gives a general representation of the processes involved in microbial fermentation.

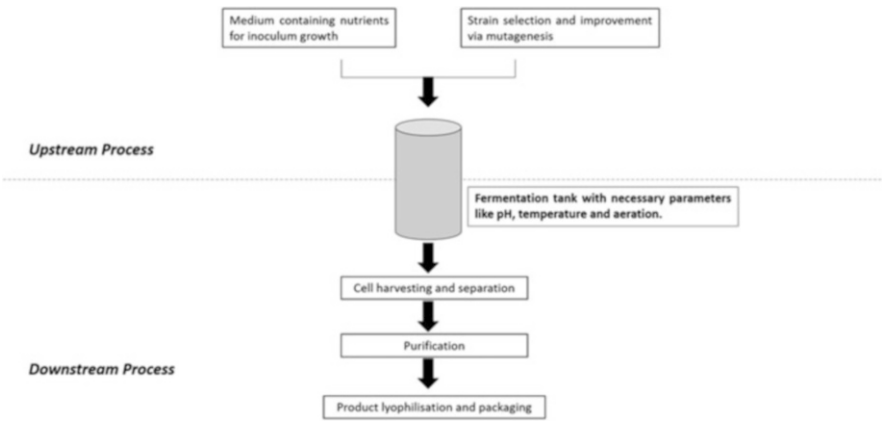

Fig. 2.1 Steps involved in microbial fermentation

2.3 Products of Microbial Fermentation

Bacteria produce metabolites like enzymes, antibiotics, organic acids, and other products of high value. Production of these metabolites commercially is a boon to mankind due to their applications in various industries like pharmaceuticals, food, and agriculture among many others.

2.3.1 Enzymes

Enzymes are biological catalysts that aid in the conversion of one substance to another without undergoing any transformation themselves (Renneberg et al. 2017). Bacteria, yeasts, and fungi manufacture and excrete enzymes into the medium that they are growing in. Usually, the enzyme invertase that is extensively used in the food industry is isolated mainly from baker's yeast or *Saccharomyces cerevisiae*. Bacterial invertases are less commonly used; however, there are many reports of invertase enzymes that have been isolated and expressed in bacterial cells as confirmed by Yamamoto et al. (1986) and Zhou et al. (2016). *Streptomyces* sp. ALKC 8 was reported to produce high levels of extracellular invertase grown in a medium containing 2% sucrose (Kaur and Sharma 2005). Factors such as pH, temperature, activators/inhibitors, mobilization/immobilization, and substrate choice determine the efficiency of invertase (Lincoln and More 2017). Another enzyme that is frequently used in the food industry for the production of syrups and alcohol and in the baking industry is microbial amylase (Gopinath et al. 2017). Dash et al. (2015) isolated a soil bacterium *Bacillus subtilis* BI19 that showed immense potential for amylase production. Similarly, Abd-Elhalem et al. (Abd-Elhalem et al. 2015) who isolated *Bacillus amyloliquefaciens* from the rhizosphere of Egyptian clover plants found that amylase production was enhanced when grown in a medium containing agro-industrial residues as carbon sources. A thermotolerant isoamylase was recently isolated, characterized, and cloned from the thermophilic bacterium *Bacillus* sp. by Li et al. (2013). Chaturvedi et al. (2014) demonstrated the production of kertinases using the chicken feather as substrate by multifunctional strains of *Pseudomonas stutzeri*, and the enzyme was then subjected to dehairing application. Proteases are hydrolytic enzymes belonging to the largest group of enzymes and are the most commercially applicable in many industrial sectors like detergent and food and also in research studies (Mótyán et al. 2013). Proteases isolated from microbial systems are of three types: acidic, neutral, and alkaline. Alkaline proteases either contain a serine center (serine protease) or are of metallo-type (metalloprotease) mainly utilized in detergent industries. *Bacillus* RV.B2.90 was found to be capable of producing protease possessing special characteristics such as being highly alkalophilic, moderately halophilic, and thermophilic and exhibiting the quality of a thermostable protease enzyme (Mukherjee et al. 2008; Gupta et al. 2002; Vijayalakshmi et al. 2011). A marine bacterium SD11 isolated from sea muds was reported to have protease activity following which the enzyme was isolated and purified. This purified enzyme had stability over a broad range of pH (7.0–11.0) and

was tolerant to organic solvents, anionic surfactants, and even hydrogen peroxide, thus implicating a potential for industrial use (Cui et al. 2015). Xylanases are also another class of highly valued enzymes with utility in various industrial sectors. *Bacillus* sp. are potent xylanase-producing bacteria with several studies having isolated bacteria from extreme environments. Thermophilic *Bacillus* strain D3 synthesizes xylanase that is active and stable over varying ranges of pH and temperature (Harris et al. 1997). *Sulfolobus solfataricus*, a thermophilic archaeon, possessed membrane-associated xylanase, which showed significant temperature and pH stability with an optimum of 90 °C and 7.0 (Cannio et al. 2004).

2.3.2 Antibiotics

Antibiotics are low molecular weight compounds produced by certain microorganisms to inhibit the growth of other microorganisms by disrupting their normal metabolic activities (Raaijmakers et al. 2002). They are secondary metabolites that are produced by microbes and were first discovered by Alexander Fleming in 1928 when he observed that the growth of the bacterium *Staphylococcus aureus* was being inhibited by a fungus contaminant growing on the same plate, which was eventually identified as *Penicillium notatum* and the antibiotic as penicillin (Tan and Tatsumura 2015). Mycelial Gram-positive bacterial genera such as the *Streptomyces* have been remarkably useful in the industrial production of almost 80% of antibiotics, such as streptomycin, chloramphenicol, neomycin, tetracycline, nystatin, vancomycin, and kanamycin (De Lima Procópio et al. 2012). β-lactam antibiotics like penicillins (ampicillin, amoxicillin, floxacillin, etc.), cephalosporins, and carbapenems are other classes of antibiotics that are manufactured on an industrial scale (Elander 2003).

The ongoing crisis of antibiotic resistance in bacteria has put tremendous pressure on developing modified and more effective forms of antibiotics that are bacteriostatic or bactericidal. Antibiotic-resistant pathogens include the methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), drug-resistant *Streptococcus pneumoniae*, drug-resistant *Mycobacterium tuberculosis*, and drug-resistant *Neisseria gonorrhoeae* among several others (Ventola 2015). Newer semisynthetic drugs have been developed keeping in mind the type of pathogen and their mode of action. Plazomicin sulfate (Zemdri) is a bactericidal drug that targets *Enterobacter cloacae*, *Escherichia coli*, *Proteus mirabilis*, or *Klebsiella pneumonia*. Recarbrio, a combination of imipenem (penem antibacterial), relebactam (β-lactamase inhibitor), and cilastatin (dehydropeptidase inhibitor), works against *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, and a few strains of *Bacteroides* (Andrei et al. 2019). Due to their outer membrane envelope, Gram-negative bacteria have a better mechanical barrier against antibiotics; therefore there is a pressing need to modify existing or develop new antibiotics that can break through these barriers. Darobactin, a new antibiotic, showed an inhibitory effect in vivo and in infected mice against Gram-negative bacteria like β-lactam-resistant *Klebsiella pneumoniae* and

Table 2.1 List of antibiotics produced by bacteria and their functions

Bacteria	Antibiotic	Target and mechanism of action
Amycolatopsis orientalis	Vancomycin	Gram-positive bacteria; Prevents transpeptidation reaction in synthesis of peptidoglycan
Streptomyces sp.	Aminoglycoside	Gram-positive and gram-negative bacteria; binds with 30S subunit to interfere with protein synthesis
Streptomyces sp.	Tetracyclines	Gram-positive and gram-negative bacteria; binds with 30S subunit to interfere with protein synthesis
Saccharopolyspora erythraea	Macrolides	Gram-positive and gram-negative bacteria; binds with 50S subunit to interfere with protein synthesis
Streptomyces venezuelae	Chloramphenicol	Gram-positive and gram-negative bacteria; binds with 23S rRNA of 50S subunit to interfere with protein synthesis
Amycolatopsis rifamycinica	Rifampin	Mycobacterium and gram-negative bacteria; inhibits bacterial DNA-dependent RNA polymerase
Paenibacillus polymyxa	Polymyxin B	Gram-negative bacteria; disrupts cell structure and integrity By binding to LPS in the plasma membrane

polymyxin-resistant *Pseudomonas aeruginosa* (Imai et al. 2019). Antibiotic hybrids are a new approach of administering antibiotics to drug-resistant pathogens. An antibiotic hybrid consists of a fusion of biologically active molecules or pharmacophores to generate a combined antimicrobial effect against the pathogens. Tobramycin-moxifloxacin hybrid, cadazolid, CBR-2092, and MCB-3681 are a few hybrids of antibiotics that have reportedly shown antimicrobial effects against some selected pathogens (Domalaon et al. 2018). Table 2.1 displays a list of antibiotic groups sourced from bacteria.

2.3.3 Organic Acids

Organic acids are extensively used in food industries as preservatives, antimicrobial agents, and acidity regulators. Moreover, organic acids also serve as the building block of chemicals for other industries. For example, succinic acid is a multipurpose chemical being used in the food industries as acidity regulators, detergents, and chelators and even in pharmaceutical industries that are usually sourced from petrochemical industries (McKinlay et al. 2007). A few examples of succinic acid-producing bacteria include *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Corynebacterium glutamicum*, and *Escherichia coli* (Lee et al. 2011). Additionally, the cost of using sugar as substrate makes it an expensive process for industries to manufacture organic acids. Microbial fermentation of agro-wastes to produce organic acids is a newer and sustainable approach that is cost-effective and efficient as well due to their high cellulose and starch content (Singh nee' Nigam 2009; Kim et al. 2018). Tammali et al. (2003) studied *Clostridium lentocellum* SG6 for its ability to ferment cellulose to acetic acid

and concluded that the strain SG6 was able to convert cellulose into acetic acid at high substrate concentrations and in just a single step. This finding could be further exploited for the large-scale production of acetic acid. The manufacture of other materials such as biodegradable plastic polylactide from lactic acid has also been reported (Jim Jem and Tan 2020). Lactic acid bacteria (LAB) like *Lactobacillus salivarius*, *L. amylophilus*, *L. helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. fermentum*, *L. acidophilus*, *L. parabuchneri*, and *L. reuteri* have been widely used in the commercial production of lactic acid from various carbon sources such as beet molasses, vine-trimming wastes, cassava starch, and whole wheat flour (Kotzamanidis et al. 2002; Roble 2003; Bustos et al. 2004).

2.3.4 Amino Acids and Insulin

The first-ever discovery of glutamic acid production by *Corynebacterium glutamicum*, previously known as *Micrococcus glutamicus*, catapulted the commercial production of amino acids. Under special growth conditions like biotin limitation, the addition of β-lactam antibiotics, and fatty acid esters, production of glutamic acid was enhanced in *C. glutamicum* (Kinoshita et al. 1957). Other glutamate-producing bacteria include *C. efficiens*, *Brevibacterium flavum*, *B. lactofermentum*, *B. divaricatum*, and *Pantoea ananatis* (Su and Yamada 1960; Hirasawa and Shimizu 2016). Veldkamp et al. (1963) reported the biotin-dependent bacterium *Arthrobacter globiformis*, which displayed enhanced production of glutamic acid under sub-optimal growth conditions. *C. glutamicum* is also utilized in the production of glutamate-derived amino acids like gamma-aminobutyrate (GABA), L-ornithine, L-citrulline, L-proline, and L-arginine which have important applications in pharmaceutical, food, and cosmetics industries (Wendisch 2014).

Rapid advancements in molecular biology and recombinant DNA technology have enabled the heterologous expression of human proteins in bacteria as carried out by Genentech in 1978 that developed Humulin, biosynthetic human insulin (Chance and Frank 1993). In this approach, the genes encoding for chains of human insulin A and B were expressed in *E. coli*, following which the enzyme chains are purified and then co-incubated to generate a functional insulin protein (Baeshen et al. 2014). Alternatively, Eli Lilly and Company expressed a single cDNA chain encoding for the human proinsulin in *E. coli* cells following which purification and cleavage of the protein chain were done to yield a bioactive insulin molecule (Walsh 2004). The latter approach is more feasible for large-scale commercial production in terms of the usage of fewer fermentation tanks than the former, which requires a co-incubation step. In recent times, even transgenic plants have been used for the expression of human insulin (Nykiforuk et al. 2006).

2.3.5 Bioethanol

Ethanol is traditionally manufactured by distillation of crops like wheat, sugarcane, sugar beet, and corn either by chemical synthesis or by microbiological fermentation using highly productive yeast species like *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Saccharomyces anamnesis*, *Schizosaccharomyces pombe*, *Candida utilis*, and *Kluyveromyces* sp. (Küüt et al. 2019). The utilization of agro-wastes like sugarcane bagasse is gaining popularity across the world owing to the sustainability of the method in addition to the high carbohydrate content in bagasse, which can be further acted upon by microbes. Usually, ligninolytic, cellulolytic, and xylanolytic bacteria are used for delignification and hydrolysis of complex carbohydrates into fermentable sugars in a process called saccharification and eventually fermentation to form ethanol (Verma and Madamwar 2003; Ire et al. 2016; Agrawal et al. 2018; Bhardwaj et al. 2019, 2020; Kumar and Verma 2020). A study reported the use of lactic acid bacteria (LAB) for the production of bioethanol from waste materials prepared from a mix of corn, corncob, paper, and pinecones. LAB isolated from fermented meat products and the facultative anaerobic *Lactobacillus plantarum* M24, in particular, showed a significantly high yield of bioethanol in their study (Soleimani et al. 2017). *Zymomonas mobilis* is another important ethanol-producing bacterium, which utilizes the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) encoded by *pdc* and *adh II* genes, respectively. Recombinant *E. coli* was developed in several studies where these two genes were expressed to enhance ethanol production from the saccharification and fermentation process of bagasse (Piriya et al. 2012; Fithriani Suryadarma and Mangunwidjaja 2015).

2.3.6 Bioinsecticides

Insect pests wreak havoc on agricultural and horticultural plants and spread diseases like malaria and dengue. Modern agriculture requires the use of pesticides like insecticides, fungicides, and herbicides for the improvement of crop growth and yield by controlling pest infestation. Chemical pesticides have been in use for decades, but the trailing consequences are drastic and harmful for terrestrial and aquatic ecosystems (Aktar et al. 2009). Moreover, there are incidences of pesticide resistance among pathogens, which is a matter of great concern (Curutiu et al. 2017). *Bacillus thuringiensis* is a well-studied entomopathogen that is antagonistic against members of the Lepidoptera, Diptera, Coleoptera, and Hymenoptera by way of its toxin called the Bt toxin (Cry protein). The genes encoding for this toxin have been incorporated into many economically important crops like cotton, corn, and brinjal (Bravo et al. 2012). Other bacterial species with insecticidal properties that have been commercialized include *Bacillus sphaericus*, *B. popilliae*, *B. thuringiensis*, *Pseudomonas aureofaciens*, *P. alcaligenes*, *Serratia entomophila*, *Streptomyces avermitilis*, *Clostridium bifermentans*, and *Saccharopolyspora spinosa.* Commercial bioinsecticides are formulated in a way that considers various aspects like the

microbial agent being used (bacteria, fungi, or nematode), the target pest, environment conditions, and application target (water, soil, or plant) (Behle and Birthisel 2014).

2.3.7 Other Products and Applications

Cancer therapeutics is ever-evolving, and in recent times, the use of bacteria as an antitumor agent has garnered widespread attention in cancer research. In the tumor microenvironment, the cells exist in a hypoxic state, which attracts facultative or obligate anaerobes like *Bifidobacterium*, *Clostridium*, *Escherichia coli*, *Listeria*, and *Salmonella* species (Duong et al. 2019). Overgrowth of these bacterial species in the tumor masses induces tumor regression by inducing apoptosis as in the case of *Salmonella* spp. (Liu et al. 2016) or by inducing high reactive oxygen species (ROS) levels in the cells as demonstrated by *Listeria* spp. (Kim et al. 2009). Some bacteria-derived anticancer agents include diphtheria toxin, botulinum toxin, exotoxin A, arginine deiminase, azurin, and plantaricin. The disadvantage of using live bacteria or bacteria-derived products is the possibility of systemic infection due to increased dose, DNA mutation, and incomplete lysis of tumor cells (Baindara and Mandal 2020).

Vitamins are organic compounds required for the normal functioning of an organism but which the body does not synthesize. Usually, vitamins are plant-derived, but microbial-derived vitamins are also being manufactured at a large scale for the consumption of domestic animals (Revuelta et al. 2016). A large number of fungi and bacteria industrially produce riboflavin or vitamin B2, an important water-soluble vitamin required in many biochemical processes of most organisms. *Clostridium acetobutylicum*, *Bacillus subtilis* PRF93, *Corynebacterium ammoniagenes*, *Lactococcus lactis* subsp. *cremoris* NZ9000, and *Corynebacterium glutamicum* KCCM11223P among many others are important riboflavin-producing bacteria (Averianova et al. 2020).

Pigments are important constituents utilized in the manufacturing of dyes and cosmetics, pharmaceutical, and food industries. Bacterial pigments are in high demand in current times due to their eco-friendly aspects as well as the ease in bacterial propagation and pigment extraction (Abdulkadir 2017). Red pigments like astaxanthin, prodigiosin, rubrolone, and undecylprodigiosin are produced by *Agrobacterium aurantiacum*, *Serratia marcescens*, *Pseudoalteromonas rubra*, and *Streptomyces* sp., respectively. Similarly, other pigment-producing bacteria consist of *Pseudomonas* sp., *Xanthomonas oryzae*, *Flavobacterium* sp., *Paracoccus* sp., *Corynebacterium insidiosum*, *Chryseobacterium artocarpi*, and several others (Venil et al. 2020).

2.4 Prospects for India in the Industrial Microbiology Sector

India has a total population of 1.3 billion out of which almost half of the population belong to the age group <25 years, representing a significant talent pool in the country (Census of India 2001). Although there is no dearth of potential, sometimes there exist discrepancies between academia and industries due to the occasional lapse in collaborative efforts from both ends. To bridge this gap, the Government of India has set up a nonprofit, public sector enterprise, the Biotechnology Industry Research Assistance Council (BIRAC), that works to promote innovative research with potential industrial applications. This objective is fulfilled by providing capital to Indian biotech start-ups and providing incubation support to individuals or groups to foster entrepreneurial ideas and to strengthen the biotech innovation ecosystem in the country (Sahoo 2020).

According to the India Brand Equity Foundation (IBEF), there are close to 3000 start-ups in the country in the year 2021, and it is estimated to cross 10,000 in the next 3 years (IBEF 2019). With increasing incidences of disease pandemics, biopharmaceutical industries are at an overdrive for the production of efficient drugs and vaccines. About 85% of the worldwide vaccine sales revenue is generated by countries like India, China, and Brazil (Douglas and Samant 2018). Biologic drugs derived from humans, plants, animals, and microorganisms comprise vaccines, blood and blood products, gene therapy, stem cell therapy, monoclonal antibodies, and others. These drugs present fewer adverse side effects as compared to invasive surgeries and small molecule therapeutics. Biosimilars on the other hand are molecules that are highly similar to the active ingredient of an approved biologic also known as the "reference medicine." These two classes of drugs have been successfully administered for the treatment of various cancers, diabetes, and autoimmune disorders (Patel et al. 2015). As a whole, the Indian biotech sector accounts for just 2% of the global biotech market, but with more investments, it is rapidly gaining traction and growing at a steady rate. It is estimated that the biologics and biosimilars industry is all set to account for USD 12 billion in the global market by the year 2025 (Biotechnology Industry Research Assistance Council (BIRAC) 2019). At present, there are more than 400 biotechnology companies in India, some of which are listed in Table 2.2.

2.5 Conclusion

Microbes are beneficial for the optimal functioning of different ecosystems due to the crucial roles they play in biogeochemical cycling, decomposition of dead organic matter, and other processes like the fermentation of food and beverages. Advancements in science and technology have made it possible to utilize these bacteria for the mass production of useful metabolites by upscaling them in bioprocess facilities. The significance of these microbial products in the well-being of humankind is immense and widely acknowledged, and industries in the biotechnology sector have relied on this fact to extract and develop purified products from

Table 2.2 List of top 20 biotechnology companies of India which use bacterial system among others for products and business

S. no.	Company	Location	Business
1	Biocon	Bangalore	Pharma/biologics/biosimilars
2	Serum Institute of India ltd	Pune	Vaccines/biologics
3	Jubilant life sciences limited	Noida	Life science ingredients
4	Syngene international ltd	Bangalore	Contract research organization (CRO)
5	Biological E.	Hyderabad	Biologics, small molecules
6	Nuziveedu seeds	Hyderabad	Agribiotech
7	AstraZeneca pharma India ltd	Bangalore	Biopharmaceuticals
8	Maharashtra hybrid seeds company (Mahyco)	Mumbai	Agribiotech
9	Bharat biotech	Hyderabad	Vaccines and biotherapeutics
10	GlaxoSmithKline Pharmaceuticals ltd	Mumbai	Pharma/biosimilars
11	Anthem biosciences	Bangalore	Contract research organization (CRO)
12	Metahelix life sciences	Bangalore	Agribiotech
13	Advanced enzymes	Thane	Enzymes and probiotics
14	Concord biotech	Ahmedabad	Active pharmaceutical ingredient (API), biopharmaceuticals
15	Panacea Biotec	New Delhi	Pharma/biosimilars
16	Ankur seeds	Nagpur	Agribiotech
17	Dr. Reddy's laboratories	Hyderabad	Pharma/biosimilars
18	Wockhardt ltd	Aurangabad	Pharma/biosimilars
19	Novozymes	Bangalore	Enzymes
20	Shantha biotechnics ltd. (Sanofi)	Hyderabad	Biosimilars

various beneficial microorganisms that can cater to various needs. Recombinant DNA and CRISPR-Cas technologies have simplified the selection and isolation of superior microbial strains while simultaneously aiding in the expression of desired genes in easy-to-culture microbial isolates (Khan et al. 2016; Tian et al. 2017). It is imperative to reiterate the importance of research and development to promote and enhance newer and better findings that could take the Indian industrial biotech sector to greater heights in the global scenario.

Competing Interests All the authors declare that they have no competing interests.

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Industrial Perspectives of Fungi

3

Susmita Paul and S. R. Joshi

Abstract

Fungi are a group of organisms that is a treasure to applications in biotechnology. Exploration of fungal biodiversity along with devising ways to maintain the collection of living fungi bears invaluable economic potential paving way for the discovery of organisms with new industrial utility that have the potential for new products. Human beings have been utilizing the benefits of a large number of fungal products manufactured using industrial processes. Metabolites and enzymes are some of such popular products apart from food. Utilized conditions that suit for the fungal growth comprise physical and chemical conditions which play a vital role in the proper growth and formation of the desired product(s) by the fungal cells. The present chapter presents some of these product formations by a variety of fungi as well as their production on large scale. The fungal products are exceptional in their own way and there are generally no other known methods for their production. Processes using certain fermentation techniques are described by a few of the metabolites produced by industry.

Keywords

Fungi · Biotechnology · Biodiversity · Metabolites · Fermentation

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_3

Abbreviations

C18	Carbon 18
Da	Dalton
EMP	Embden-Meyerhof-Parnas pathway
GMO	Genetically modified organisms
SmF	Submerged fermentation
SSF	Solid-state/substrate fermentation;

3.1 Introduction

Mycelial fungi have been and are used by industry in the generation of different products of human use. These varied products are produced by a variety of fungi and are generally produced in industries in large amounts as metabolites, foods, and various enzymes (Rokem 2010; Agrawal and Verma 2020a, 2021; Agrawal et al. 2018). A variety of environmental conditions not only influence but also promote the growth of fungi (Calvo et al. 2002). The competence of these cells to amass the desired product(s) depends mostly on the physical and chemical conditions used for fungal propagation (Waites et al. 2001). There is a great potential for these filamentous fungi in terms of economic aids as well as their use to produce novel items. The use of such organisms in industrial processes is of huge economic significance (Hyde et al. 2019). These products are limited, and characteristically if it is checked, then there are no other commercial means to produce these products. Fungi carry several distinct characteristic features which make them diverse from other life forms. This growth of these fungi is in a filamentous branching mode by means of apical growth and lateral branching, having heterotrophic nutrition (Naranjo-Ortiz and Gabaldón 2019). The life cycle of fungi commences by the propagation of their resting structure or spore. In vegetative growth, the biomass increases due to the utilization of substrate by the growing cells leading to the formation of hyphal structures (Bobek et al. 2017). Then a porous three-dimensional net which is known as mycelium is formed from the fungal hyphae. The sporulation of fungi during the life cycle is characterized by the appearance of distinct morphological structures which look different from the fungal mycelium. Generally, this happens when there is a lack of nutrients (Adams et al. 1998).

This spore formation is not initiated in all filamentous fungi. It is typically the poor nutrient conditions that don't favor the formation of spores. When the nutritional conditions are favorable and proper, spores are formed and shaped. Furthermore, there is no evidence of any sexual cycle in filamentous fungi as these fungi are known to develop while growing in the lagging phase of the life cycle. In contrast to this, a sexual cycle known as the perfect stage in the life cycle of a fungus is observed in others which, of course, is dependent on the precise nutritional conditions.

Table 3.1 Products and product uses of filamentous fungi

Products	Applications
Antibiotics	Brewing industry, biological control agents
Alkaloids	Baking process, biobleaching
Enzymes	Mushroom production, coal solubilization
Ethanol	Cheese-making process, bioremediation
Gibberellins	Oriental food fermentations, dyes/dye intermediates
Organic acids	Mycorrhizal inoculants, Quorn myco-protein
Vitamins	Waste treatment, Quorn myco-protein

The filamentous fungi perform a significant function in the living sphere of the Earth by the cycling of materials and minerals that are made available by the decomposition of organic matter which includes both macro- and micronutrients (Gougoulias et al. 2014). These fungi are exploited for numerous purposes such as the production of fungal metabolic products especially the metabolites for use in food and food products. There is a great variety of products produced by fungi especially the filamentous fungi which include a wide range of fungal metabolites (Table 3.1).

The growth conditions of each specific fungus are characterized systematically which is meant for the manufacturing of desired goods. The potential of any fungal metabolite and its production using fungi begin with basic research on its isolation and growth and thus can be upscaled to its full-scale utilization to industrial progression subsequently (Nielsen and Nielsen 2017). Every researcher of industry who undertakes this research on fungi having potential product formation is kept confidential, and hence the details are not available to the masses, and if available too, it will be in a patented form. Thus, it is unlikely that the specific information for the process will be available to everyone. Here, the information for particular metabolites where additional comprehensive data is accessible on their industrial production is more focused. There are a plethora of fungal products which are described as a few examples in this section. This description is presented giving the procedures involved in their production at an industrial scale. Some of the products that are known today and produced industrially are routine, but the details on the techniques involved in the process are not freely obtainable (Bennett 1998).

There are heterologous proteins that are produced using the filamentous fungi at an industrial scale and have wide commercial applications. On the other hand, products from higher fungi have wide applications in therapeutics and the food industry (Ward 2012).

3.2 Fungal Products

There are an enormous variety of metabolites produced by industrially important filamentous fungi. Diverse collections of compounds are described here with the intent to give an illustrative performance. Small molecular weight (> 1000 dalton)

compounds are one group, which are called "metabolites." These metabolic products can be primary or secondary metabolites. The description of these metabolites is more of biological nature rather than chemical. Such basis is linked to their density, stereochemistry, and chiral properties. The chemical means of production of these metabolites from the selected fungi is cost-intensive and would lead to the production of the more costly product, and hence biological means are adopted for their large-scale production (Waites et al. 2001).

3.2.1 Metabolites

One of the plant growth stimulators, which is shaped by mounting the fungus *Gibberella fujikuroi* on solid substrates, is gibberellic acid belonging to gibberellins which is a widely used metabolite. This growth promoter is known to enhance growth and maturation by cell elongation as well as cell division which subsequently causes early seed germination (Hedden and Sponsel 2015). The growth of several crop plants belonging to field crops, fruits, and vines along with various ornamental plants is promoted by gibberellic acids. The red pigments that are produced by the yeast, *Monascus* species, are made by growing this yeast in bread or rice grains. The production of red pigments by this yeast is now achieved by submerged fermentation which has considerably substituted the traditionally used solid-state fermentation method. Antibiotics represent a group of several economically stimulating metabolites. Other fungal metabolites such as lovastatin (mevinolin) obtained from filamentous fungi have diverse use in therapeutics, and it belongs to the class of statin drugs used for lowering cholesterol (Juzlovâ et al. 1996). Statins as clinical drug were initially accepted by the Federal Drug Administration in 1987, and it has a market value of about $15 billion per year based on the drug requirement projections. The statins are produced using filamentous fungi using the submerged fermentation method (Manzoni et al. 1999).

3.2.2 Enzymes

The key benefit that is offered by the majority of enzymes is that they are highly active at atmospheric conditions when compared to the increased temperature and pressure that are essential for numerous chemical reactions executed in the industry. The catalytic competence of the enzymes is significant, and the quantity required is much less in biologically induced reactions in contrast to chemical reactions. A wide variety of reactions can be completed by enzymes that offer high versatility in their activity (Robinson 2015). The novelty in their performance and activity are looked for and assessed in enzymes that can make them well-suited for sustainable utilization. Enzymes are produced by diverse cells and among them, filamentous fungi are the most prominent. Industrially important filamentous fungi produce enzymes used by industry for numerous purposes (Table 3.2). A variety of beneficial characteristics

Table 3.2 Few representative fungi and their enzymes of commercial importance

Fungi	Enzymes
Aspergillus Niger	Amylases, catalases, cellulases, glycoamylases, glucose oxidases, hemicellulases, lipases
Aspergillus oryzae	Amylases, glycoamylases, hemicellulases, lipases
Penicillium spp.	Catalases, dextranases, asparaginases, glucose oxidases
Trichoderma reesei	Cellulases

are acquired by the enzymes that shape them perfectly as catalysts in a great diversity of reactions.

The manufacturing process of each of the enzymes is different although produced by fungi of different categories. In this section, detailed information on the manufacturing process of glucoamylase is presented. The huge volume product, glucoamylase enzyme, is used industrially for the production of starch and sugar. The glucose produced in the process can then be converted to fructose syrup by using the enzyme glucose isomerase (Raveendran et al. 2018).

Glucoamylase happens to be an important industrial product and its production is carried out using solid-state fermentation. Submerged fermentation may also be utilized for the production of this enzyme on an industrial scale. Chymosin and rennet belonging to enzyme group rennin are utilized in cheese production as they bear the properties of milk clotting. The calf stomach especially the lining of the fourth stomach was the source of this enzyme. There have been numerous alternate sources of rennin today that have been discovered and used industrially since the calf stomach was not sufficient for its supply to meet the industrial requirements. Today, a large number of filamentous fungi mostly belonging to *Mucor* species are one good source of rennin, and its production is undertaken by utilizing the submerged fermentation method (Rokem 2010).

When it is compared to chemical catalysts, enzymes often have a competitive advantage. The enzymatic method is ecologically responsive, as it needs mild environments and does not usually lead to the manufacture of toxic by-products (Chapla et al. 2013). Enzymes are employed to catalyze reactions in manufacturing procedures of numerous divisions together with industrial bioconversion (biocatalyst), environmental bioremediation, agricultural subdivisions, and also biotransformation of several compounds, for example, flavonoids (Das and Rosazza 2006). There are numerous origins of enzymes which include the sources through plants, animals, and the microorganisms, such as bacteria, fungi, and protists. Enzymes from microbes have commonly been utilized for the reason that they can be easily isolated in large quantities, have low production cost, and have consistency at extreme environments and the related compounds are manageable and not much harmful. Enzymes from microbes that are secreted into the media are extremely consistent for manufacturing progressions and in industrial applications. Microbes which are isolated from diverse bases from species and strains in spite of being from the same genus might yield variable stages of enzymes of divergent properties (Nigam 2013).

Enzymes from fungi are recognized due to their large number of applications. This is also for the reason that fungi are capable of being grown on affordable materials and secrete greater quantities of enzymes in the culture medium, which simplifies the process of downstream processing (Anitha and Palanivelu 2013). Numerous enzymes from fungi can be obtained economically which include the enzymes such as cellulases, amylases, lipases, proteases, and xylanases (Cheng et al. 1999; Agrawal et al. 2019; Bhardwaj et al. 2019a). The utilization of enzymatic reactions as an alternative to organic solvents or chemical reactions is industrially important. There are several enzymes of fungal origin and the sources of enzymes that are known to be employed in many applications, but limited fungal types meet the standards for production at an industrial scale (McKelvey and Murphy 2017).

Several enzymatic applications related to the food industries have become attentive toward hydrolytic reactions (Raveendran et al. 2018). The production of prebiotics involves enzymes like glycoside hydrolases and β-galactosidase. These prebiotics are dietary substances that are collectively made of oligosaccharides and non-starch polysaccharides. For example, inulin, fructo-oligosaccharides, lactulose, and galacto-oligosaccharides, which particularly encourage the growth of advantageous intestinal microorganisms in humans, are a few of the compounds included under the category of prebiotics (Chowdhury et al. 2015). Cellulases, pectinases, and xylanases are extensively employed for the processing of wines and juices. Amylases, in the starch liquefaction method, hydrolyze starch and transform it into glucose syrups. Tannases are employed to reduce tannin levels in food products like tea, fruit juices, and wines. Another important application is that such enzymes are employed in hydrolyzing esters of gallic acid to harvest gallic acid, which acts as a substrate in the process of making food preservatives (Verma et al. 2018).

As a supporting agent, starch is utilized in the de-sizing method to avoid the disintegration of the warp thread which is employed in the textile industry, during the weaving process. In this application, amylases produce products that do not degrade the textile fibers. Modifications and changes in the starch contained in coated paper and textiles are brought about by the enzymes α-amylases for use in the pulp and paper industry. In the process, during processing and finishing, the textiles quality and de-sizing starch coated paper is safeguarded which may get distorted or destroyed during the mechanical process. One of the eco-friendly approaches is the recycling of waste paper in the paper industry, and the utilization of enzymatic reaction of, lipases, cellulases, and xylanases which assist in the elimination of polluted ink (Bhat 2000).

In the step of polishing for clear dyeing, the use of proteases and cellulases is for refining color appearance and surface vibrancy, and also to withstand textile wrinkling. To improve the standard of the pulp by eliminating lignin and hemicelluloses, which are impurities produced in the paper and pulp industries, lignin peroxidases, laccases, xylanases, and manganese peroxidases are all utilized (Choi et al. 2015; Verma and Chaturvedi 2017; Bhardwaj et al. 2020; Verma and Madamwar 2002a; Bhardwaj et al. 2019b).

Since it is observed that chemical procedures have ill effects on the environment. Biological processes involving proteases in the processing of leather for debating

and dehairing of hides and skins are eco-friendly approaches. Tannases, which are another group of enzymes, are utilized for the superior quality of leather tannin preparation in the industry involved in the production of leather. This process of enzymatic development is environment friendly and assists to control waste resulting from industrial manufacturing activities. Proteases, amylases, and lipases are components in dishwashing detergents (de Souza et al. 2015). Among all liquid detergents, around 90% of all contain amylases, and these are considered to be the second type of enzymes utilized in the making of detergents using the enzymatic process (de Souza and de Oliveira Magalhães 2010).

Poultry and pigs, which are the monogastric animals, are incapable of totally degrading and utilizing plant-based feeds, containing substantial quantities of cellulose and hemicellulose. For such animals, enzymes like xylanases, cellulases, and β-glucanases have been utilized in the feed that is cereal-based (Bhat 2000). Phytases, when it is added to the feed, ease the release of native phosphorus which is bound in phytic acid in feed that is cereal-based for monogastrics. When it is observed in the entire plant, it was found that around 85–90% of the phosphorus is bound to phytic acid, which makes it hard for monogastrics to employ. Hence, accessible inorganic phosphates need to be improved in the feed to meet the essential concentration of the mineral in the animal feed. Phytases can lessen the external expansion of inorganic phosphorus in the feed (Kirk et al. 2002).

There are some enzymes such as laccases, dioxygenases, peroxidases, and phenol oxidases that are utilized in wastewater treatment (Durán and Esposito 2000). The decrease in organic matter, which supplements to a cleaner effluent, is one possible application of enzymatic treatments of wastewater (Kumar et al. 2019; Kumar et al. 2018a). Furthermore, white-rot fungi lead to the production of lignin-degrading enzymes like lignin peroxidases, manganese peroxidases, and laccases which are employed for degradation of the extended form of refractory organic impurities, accelerated biodegradation of polycyclic aromatic hydrocarbons (PAHs), and chlorinated hydrocarbons from the atmosphere (Gao et al. 2010; Agrawal and Verma 2020b, 2020c; Agrawal and Verma 2019).

Industrial enzymes are generally molded in bioreactors of volume up to over 200,000 liters which are then cleaned in the downstream process at an industrial scale. Fungi that are capable of getting utilized for such usages must be fast-growing, but not pathogenic nor producing any mycotoxin. To progress in the yield of production, enzymes in addition to their activity, approaches such as recombinant DNA technology, heterologous expression, and gene cloning technology can help to eliminate the complications that are normally associated with the large-scale industrial production of enzymes (Hyde et al. 2019; Kumar and Verma 2020, 2021).

3.2.3 Biomass

There are numerous foods made by modifying the taste as well as the texture of substrates by utilizing fungi which happens by the action of various enzymes. Novel foods with appealing texture and flavor are produced by growing filamentous fungi

using submerged fermentation conditions which generate a good amount of fungal biomass.

Biomass of microorganisms acts as a good source of production of certain compounds which have industrial applications. One such production of compounds includes the production of biofuel. There is some promising substitution for petroleum oil that comes from organic biomass which is the principal source for the majority of biofuels, ethanol, and biodiesel. All microorganisms cannot be a good source of oils and fats. Microorganisms invariably hold lipids for the crucial performance of membranes and membranous constitutions. Organisms that can assemble around 20% or more of their biomass as lipid have been designated as "oleaginous organisms" in keeping with oil-bearing plants that are likewise termed as "oleaginous plants." Out of a few 600 yeast species, around 25 or so can assemble more than 20% lipid, and of the 60,000 fungal species, fewer than 50 contain more than around 25% lipid in their biomass (Akpinar-Bayizit 2014).

According to the stage of fungal development, age, and state under which fungus is cultured, the respective proportions of all the lipid constituents may vary in each variety. Most of the fungal species contain, in order of quantity, oleic acid (C18), palmitic acid (C16), and linoleic acid (C18) as the major acids, with stearic acid (C18), linolenic acid (C18), and palmitoleic acid (C16) as the minor ones (Moser 2010) [C indicates the number of carbon atoms]. Lipids produced from filamentous fungi exhibit considerable potential for biofuel production. Fungi are reported to contain high percentages of total lipid in their dry biomass which act as an oleaginous criterion for fungi. Production of a range of substances by fungal biomass has found potential use in modern medicine, agriculture, and industry (Paul et al. 2020).

3.3 Fermentation

Fermentation technology includes the utilization of microbial enzymes for manufacturing compounds that have applications in energy production, material production, chemical industries, pharmaceutical industries, and food industries (Raveendran et al. 2018). Such a process also takes place naturally in several foods. Fermentation is a process that has been in use since ancient times for the preservation of foods and has been an asset for food science and technology (Fig. 3.1). It is a well-established technology for the preservation of food and production of bread, vinegar, beer, yogurt, cheese, and wine. With time, the process has got advanced and as a technology, it is diversified (Maicas 2020).

It is the biological procedure in which numerous microorganisms like yeast, bacteria, and fungi are concerned with the conversion of complex substrates into simple compounds that have wide applications for use by humans in the form of enzyme production, metabolites, biomass, recombinant technology, and biotransformation products of industrial scale. There are two key products of fermentation, which include organic acid and alcohol. The process of fermentation also results in the production of secondary metabolites such as antibiotics, enzymes, and growth factors (Anderson 2009).

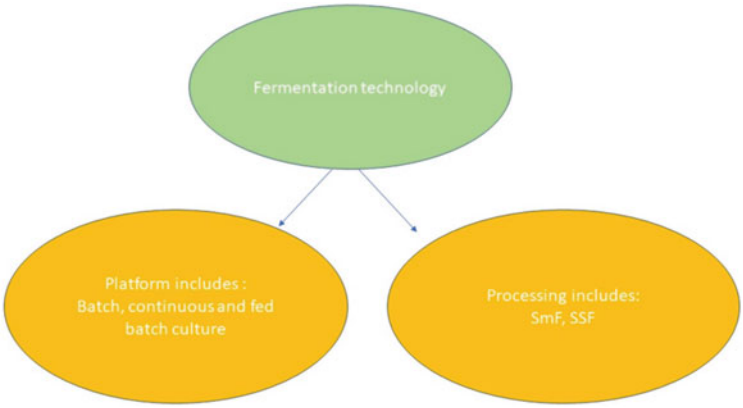

Fig. 3.1 Pictorial presentation of fermentation technology

Depending on the diverse parameters for fermentation such as environmental parameters and essential organisms, the fermentation technology has become progressive and advanced. In general, the bioreactor is essential for such a procedure which is based on the provision made to supply the growth medium can be clubbed under batch, continuous, fed-batch fermentation, and immobilization method. With regard to the obtainable amount of oxygen, mixing of substrate occurs in single and mixed culture in submerged fermentation (SmF) (Paulová et al. 2013).

3.3.1 Types of Fermentation

Fermentation processes can be categorized into the following types depending on the purpose and the consistency of the medium.

3.3.1.1 Solid-State Fermentation

Solid-state (or substrate) fermentation (SSF) is defined as fermentation that occurs in solid supporting, non-specific, natural state, and low moisture content medium. In this method, substrates such as nutrient-rich waste can be reprocessed. The solid substrates used in SSF include the bran, bagasse, and paper pulp. The fermentation of substrate takes a long time as SSF is a slow process and it allows the release of the nutrients in the fermenter tank in a controlled manner. This process involves less moisture content and it is the finest fermentation technology used for fungi and other microorganisms. Nevertheless, this procedure is not appropriate for bacteria, since this fermentation cannot be used for organisms that need high water content (Pandey et al. 1999).

3.3.1.2 Submerged Fermentation

In submerged fermentation(SmF), microorganisms require a controlled atmosphere for efficient production of good quality end products and to achieve high yield with

ideal productivity. For the production of various types of products of microorganisms growth tanks with batch, fed-batch, or continuous means are used in industrial bioreactors(Aguilar 2017).

For the production of alcoholic beverages (whisky, beer, brandy, rum, and wine), preservatives or acidifiers like lactic acid, citric acid, and vinegar are utilized in the food industry, while the flavor enhancers including monosodium glutamate or sweeteners, aspartate, and amino acid are added in appropriate quantities in the submerged batch cultivation process. There are various ways through which submerged cultivation can be carried out, which are identified by using microorganisms in bioreactors. This section provides a brief discussion on the characteristic features, advantages, and demerits of each fermentation method. Growing microorganisms in liquid medium in several types of food industrial products has been determined as the most significant application for continuous, batch, and fed-batch fermentation processes (Joshi et al. 2018).

3.3.1.3 Batch Cultivation

The batch cultivation system is a closed system that works under sterile conditions. These cultivations comprise providing inocula, nutrients, and medium which are mixed in the bioreactor, while the volume of the culture broth remains constant throughout the microbial cultivation process in such system (Yang 2019).

3.3.2 Substrates Used in Fermentation and Type of Fermentation Used for Different Biomolecule Production

Since there is a wide variation in the product of fermentation, it is very important to select a good substrate for use in the process to yield the desired product. The optimization of every substrate is necessary for this method. This is principal because of the fact that microorganisms grow differently in different substrates. The rate of utilization of different nutrients by the microorganism differs in every substrate, and consequently, the end-product formation varies accordingly. Some of the commonly used substrates in SSF are rice straw, vegetable waste, wheat bran, fruit bagasse, synthetic media, and paper pulp. Liquid media, molasses, wastewater, vegetable juices, and soluble sugar are common substrates used in SmF to extract bioactive compounds. A variety of bioactive compounds that are produced using this fermentation includes enzymes, antioxidants, antibiotics, biosurfactants, and pigments (Joshi et al. 2018).

3.3.2.1 Type of Fermentation Used for Enzyme Production

Enzyme cultivation is used for the production of a diverse group of enzymes that have wide applications. Microbes such as fungi and bacteria are invariably the major groups that are used for the production of enzymes. Such production can be achieved by submerged as well as solid-state fermentation. Enzyme production by using bacteria generally uses SmF method, for the reason that it requires greater water

potential in the medium. SSF method is applied in the case of fungi that require less water potential (Gowthaman et al. 2001).

Almost around 75% of the industries are utilizing SmF method for the production of enzymes, in the world today. One of the key reasons for using SmF over SSF is that it does not support genetically modified organisms (GMO) to the degree to which SmF can support, and hence SmF is preferred over SSF. Another purpose of using SmF is that it lacks peripheral support which is associated with the cultivation of a variety of microbes for the production of enzymes using SSF. The microorganisms used are different in SmF and SSF in their metabolism display which becomes an extremely critical deciding factor in the process. The influx of nutrients and efflux of the waste constituent is carried out in diverse metabolic regulations in the cultivation process. Even the smallest differences from the precisely optimized parameters affect the production of products also leading to the accumulation of undesirable end product (Joshi et al. 2018).

Type of Fermentation Used for Fungal Enzyme Production

Several genera of fungus such as *Aspergillus* have been isolated and characterized as enzyme producer which is industrially significant for the manufacture of many enzymes. This fungus has been recognized as a model microorganism in the manufacture of fungal enzymes and has become one of the main sources of fungal enzymes. Even while using a particular fungus for the production of fungal enzymes, the choice of fermentation process is important as SSF and SmF affect the yield of a particular fungal product (Agrawal and Verma 2020d; Kumar et al. 2018a; Bhardwaj et al. 2017; Verma and Madamwar 2002b) . For example, using the SmF method, phytase is extracted from *Thermoascus aurantiacus* which is not the case with SSF method (McKelvey and Murphy 2017).

3.3.2.2 Type of Fermentation Used for Antibiotic Production

Antibiotics are the most significant extract which can be obtained from microorganisms utilizing fermentation technology. It is a bioactive compound used for human and animal welfare. The first antibiotic which was produced using fermentation technology was penicillin from *Penicillium notatum*. The penicillin discovery is attributed to Alexander Fleming as it was first reported by him. This was commercially optimized using SSF and SmF method during the 1940s using SSF and SmF method, but currently, *Penicillium chrysogenum* isolates have been characterized as advanced yielding producers of penicillin. Aminocillins, carbapencins, monobactams, cephalosporins, and penicillins collectively are identified as β-lactam antibiotics (Midtvedt 1995). A few other antibiotics like tetracycline, streptomycin, cyclosporin, cephalosporin, and surfactin are produced from using fermentation procedure. *Streptomyces clavuligerus*, *Nocardia lactamdurans*, and *Streptomyces cattleya* produce cephamycin C from sunflower cake and cotton-de-oiled cake in which wheat raw is added in SSF system as substrates for engineering cephamycin C. Penicillin was originally produced in SSF by actinomycetes and fungi in mixed cultures (Manpreet et al. 2005).

3.3.2.3 Batch Cultivation for Biomolecule Production

It is a semi-open system where several nutrients are added aseptically in fed-batch manner with the culture/inoculum supplemented in a stepwise manner into the bioreactor. The volume of the liquid culture in the bioreactor rises within this period.

The rise in productivity, improved yield by the controlled consecutive accumulation of nutrients, capacity to attain advanced cell densities, and extended product synthesis are the key compensations of the fed-batch over batch culture system.

Enzyme or microbial cell also called "Immobilized Cell Technology Active Biocatalyst" has improved the productivity of bioprocesses which is achieved through the controlled process leading to product formation with high concentration. This is done by cell immobilization or recycling by feeding approaches in high-density cultures. Cell immobilization typically is studied in the food and gas-liquid mass. The process is done in three-phase bioreactor involving three phases of mass transfer. Such bioreactor objective has a key process amplification which is achieved by the development of gas-liquid mass transfer (Joshi et al. 2018).

3.3.3 Products from Fermentation

There are several products that can be produced through the process of fermentation having great importance (Fig. 3.2). Some of these products are described below:

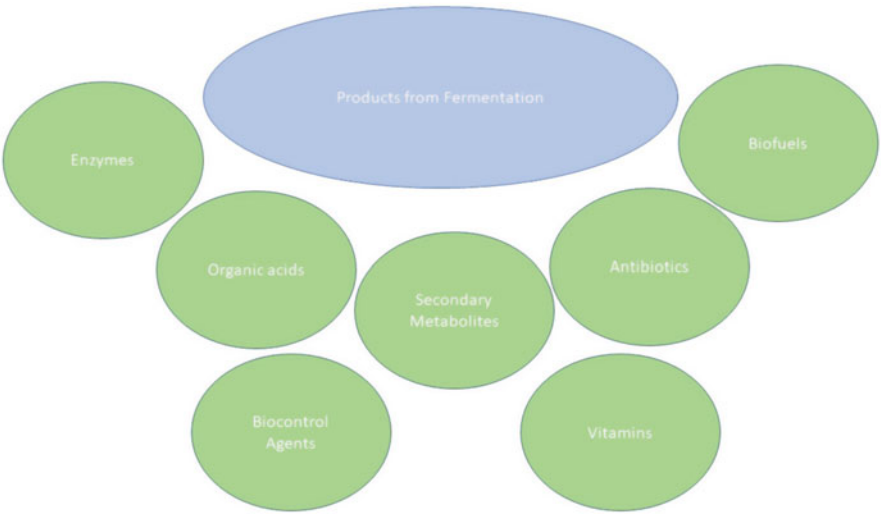

Fig. 3.2 Pictorial presentation of products from fermentation

3.3.3.1 Primary Metabolites

Enzyme Production

Agriculture, as well as industrial substrates, is considered to be exceptional resources for enzyme production in SSF. The expenses of enzyme manufacture by SmF method are high when compared to SSF. Nearly, all known microbial enzymes are shaped by this process. According to various research studies, a good amount of work has been achieved on the manufacture of the enzyme of industrial significance. Some of the representative enzymes of this category include cellulases, lipases, proteases, glucoamylases, amylases, ligninases, xylanases, pectinases, and peroxidases. Thermostable enzyme-like xylanase from a thermophilic bacterium, *Bacillus licheniformis*, has been reported to be produced using this process. Enzymes produced from such a process are found to be more thermostable than SmF method. It is found to be 22-fold higher in SSF than in SmF system from the open xylan agar plate. Promising xylanase-producing bacterial strains like *Bacillus pumilus* are characterized to produce the enzyme from both the processes (SmF and SSF fermentation). *Rhizopus oligosporus* is utilized to harvest acid protease from rice bran with no evidence of the production of toxin during the process using SSF (Kupski et al. 2012).

Organic Acids

Several organic acids produced by SSF include gallic acid, citric acid, fumaric acid, kojic acid, and lactic acid. A few of the agro-industrial wastes that are ingenious substrates for the production of citric acid in SSF include wheat bran, de-oiled rice bran, sugarcane, carob pods, coffee husk, kiwi fruit peels, pineapple wastes, grape pomace, and apple. Pineapple waste is usually utilized as a substrate during the production of citric acid from *Aspergillus*. For the manufacture of lactic acid from *Rhizopus oryzae*, sugarcane bagasse impregnated with glucose and $CaCO_3$ is utilized (Soccol et al. 1994).

Biocontrol Agents

Since there are different modes of action of biocontrol agents and based on this, fungal agents are found to have more potential to perform as such agents. For example, control of mosquitoes, *Lagenidium giganteum*, is carried out by utilizing a fungal agent. These organisms function by encysting the larvae of mosquitoes. Here the fungal biocontrol agent uses larvae as a substrate for growth (Scholte et al. 2004).

Vitamins

There are a few water-soluble enzymes such as nicotinic acid, vitamin B_{12}, thiamine, riboflavin, and vitamins B_6. These are produced on SSF with the help of different species of *Rhizopus* and *Klebsiella*, which are well-known producers of vitamin B_{12} (Keuth and Bisping 1994).

3.3.3.2 Secondary Metabolites

Some fungi are capable of producing secondary metabolites such as gibberellic acid in their stationary phase of growth. Gibberellic acid as a secondary metabolite has its production consistently increasing in the SSF system. Increment of gibberellic acid in a study was reported to be 1.626 times greater in SSF than SmF using *Gibberella fujikuroi* in which wheat bran was used as substrate (Joshi et al. 2018).

Antibiotics

As elaborated above, cephamycin C, cyclosporin A, penicillin, neomycin, iturin, and cephalosporins are some of the common antibiotics which are produced from microorganisms using the SSF method. Penicillin is produced from *Penicillium chrysogenum* in which wheat bran and sugarcane bagasse are utilized as substrate under high moisture content (70%). *Nocardia lactamdurans*, *Streptomyces clavuligerus*, and *Streptomyces cattleya* produce cephamycin C. In SSF, antibiotic penicillin is produced from actinomycetes and fungi by mixed cultures (Joshi et al. 2018).

3.3.3.3 Biofuels

In the current scenario, ethanol is considered to be the most extensively used biofuel. While it is very easier to yield ethanol using SmF. The method is ideal and preferred due to low water requirement, little volumes of fermentation mash with the protection of end product, and low amount of liquid water disposal. It reduces pollution problems and is most frequently used for ethanol manufacture due to its availability in abundance. *Saccharomyces cerevisiae* is utilized for ethanol production in SSF with apple pomace augmented with ammonium sulfate in fermentation. Sweet potato, rice starch, wheat flour, potato starch, and sweet sorghum are some other substrates used in general (Kupski et al. 2012).

3.3.4 Production of Alcohol

Even though there is a difference between beer, wine, and liquor as well as other alcoholic beverages, these have one thing in common. This similarity lies in that all these are fermentation products produced by the use of yeast like *Saccharomyces cerevisiae* though yeast like *S. carlsbergensis* may be used for the production of beer yeasts which are fungi but are not mycelial. The yeasts are fungi possessing unicellular cells, and their reproduction happens by asexual methods like fission or budding. The well-studied reaction by which alcoholic beverages are produced is commonly described as fermentation and may be summarized as:

$$\text{Yeast} + \text{Glucose} = \text{Alcohol(Ethanol)} + CO_2 \tag{3.1}$$

This reaction is also significant in baking bread, but the product which is desired in baking is carbon dioxide rather than alcohol. The production of alcohol occurs best in the absence of oxygen. Though in the viewpoint of yeast, alcohol and carbon

dioxide yeast, alcohol, and carbon dioxide are waste products as the yeast lingers to grow and metabolize in a sugar solution, increasing concentration of alcohol will become toxic when it reaches a concentration of around 14–18%. This results in the killing of the yeast cells. This is the reason why the percentage of alcohol in wine and beer can only be about 16%. For the production of beverages (liquor) with higher concentrations of alcohol, the fermented products need to be distilled. The presence of excess glucose in *Saccharomyces cerevisiae* represses respiration. In principle, materials rich in sugars (or starches) are then fermented resulting in the production of alcohol (Pepin and Marzzacco 2015).

3.3.4.1 Alcoholic Products

Mainly two products are known in common. They are beer and wine.

Production of Wine

In the production of wine, the common species of yeasts which are used are *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Saccharomyces uvarum.*

The yeast hydrolyses sugar, usually sucrose, to pyruvic acid via the Embden-Meyerhof-Parnas (EMP) pathway and then to acetaldehyde to ethanol. Their action is exothermic where the heat is dispersed which reduces the speed of the reaction. In addition to that, up to 50% of sugar by weight can result in the production of alcohol, but the solution invariably does not go beyond 15% ethanol concentration. The reason is that the fungus is sensitive to high concentrations of ethanol present in the solution of the fermentation process. Wine is still made in a similar way as it was made centuries ago. Nevertheless, unlike beer, there is still a great deal that cannot be measured in the manufacture of wine. This will be discussed in the following paragraph. Separation of the grapes from which the wine is to be prepared is done first from the stem and then crushed for releasing the juice. The mixture of the skin, juice, and seeds is named the *must*. Grapes might be crushed using numerous approaches, from stamping on them with bare feet to the use of sophisticated electric presses. If the desired product is white wine, the free juice needs to be transferred to a fermentation container, and the peels and stems should be removed and pressed over. The juice of the second press can be further included in the original juice or used to make one lesser graded wine. If red wine is to be produced, the skins of the grape need to be added into the fermentation chamber with the juice. The red color of this wine comes from the red pigment present in the epidermis of the grape skin. Numerous vessels may be utilized as the fermentation chamber (Maicas 2020). 32-gallon plastic garbage is the most low-cost and regularly used vessel for this process.

Ideal strains of yeast are often added to the juice in the fermentation chamber, but may not always be desired. An adequate number of yeasts are found already on the skin of the grapes, hence that this step of addition of various strains of yeasts could be omitted and hence is not desired as stated above. This is one of the uncontrolled quality of wines. Subsequently, the yeasts which breed on the grapes differ in the different vineyard, specifically if they are from different climatic zones in different

countries. Thus, the quality of the finished wine will also differ which relates to the claim that one country's wine is better than another. The inclusion of the ideal yeast provides some degree of control to the end product. Sulfur dioxide is usually presented into the juice during this process to inhibit bacterial growth that might spoil the taste of the end product. Fermentation is allowed to take place for around 8 to 10 days. Subsequently, the primarily formed wine is drawn off of the skin, if the skin is still existing. Any liquid obtained from the grape skin which persists all through the fermentation process is of a deprived quality and is used in poorer-quality wines or for vinegar production (Pinto et al. 2015).

When the preliminary fermentation process is over, the liquid is allowed to ferment for 20 days to approximately a month duration. Throughout this fermentation, the dead yeast cells, as well as other particulate material, settle down at the bottom of the liquid. Once this development is completed, the wine is separated from the sediment and transferred to an aging chamber. As the aging process lasts, further sedimentation happens, and the wine is frequently transferred across a series of chambers during aging. This process is recognized as *racking*. If the final chamber is a wooden cask, this correspondingly enhances one more uncontrolled amount into the ultimate product. For the reason that wooden cask cannot be cleaned, they deliver an exceptional character to the wine, a few of which are said to make the wine "superior." The utilization of stainless-steel vats has removed this wine character ambiguity (https://bio.libretexts.org/Bookshelves/Microbiology/Book).

The aging of wine is not a constant parameter. For white wines, typically 1 year to 18 months is needed, but red wines can age for as long as 5 years. At this aging phase, the wine is experimented with and judged by a wine master. The fate of the quality designation of wine is reliant upon the verdict of the wine master. The wine might be bottled subsequently when aging is complete or used only as a mixture to make a substandard wine. The bottled wine, yet again, based on the verdict of the wine master, might be aged for an extended period in the bottles or supplied instantly after bottling. This accounts for the inconsistency of the complete wine product (Pinto et al. 2015).

Production of Beer

Ales and lager beer use cereal grains, generally barley, as the substrate for fermentation. The grain is dried and then germinated synchronously. The germination process includes the release of amylases within the grain. These enzymes are formerly utilized to digest the starch of ground cereals mixed into a mash, resulting in the release of hexoses. The subsequent wort is drained off. After several treatments, with the addition of hops, the liquid is inoculated with precise yeasts and the blend fermented for a short period.

Nowadays, beer being consumed in huge volumes, its production is mainly mechanized as in all mass-produced products. Despite the sophisticated machinery which is used in brewing beer, it is still fundamentally the identical procedure that has been used for hundreds of years. Nevertheless, beer production has turned out to be very sophisticated due to developments in the knowledge that has resulted in advancements in fermentation science. Preceding and even during the 1800s, many

knew how beer could be produced, but nobody knew the science behind each step involved in the process. This was not until the nineteenth century when it was understood that all through the germination of cereal grains, enzymes were released that not only break down the barley starch and protein into simple sugars and amino acids but also break other carbohydrates, such as potato, corn, and wheat. This awareness lowered the cost of production of beer because germinated barley is a bigger investment than the utilization of potato, corn, and wheat. It was during that period when it was recognized that yeasts were the organisms that were accountable for the fermentation process (https://accounts.smccd.edu/case/beer.html).

While the procedure of fermentation had been used for thousands of years, it was assumed to be a magical process instead of biological based on substrate usage. In the early days, several rituals and superstitions were attached to direct and control fermentation. By the seventeenth century, it was well established that yeast existed all through the fermentation process, although its role was controversial with no conclusive evidence established about the process. There were two contrasting views proposed on this subject. According to one view, yeast was essential for the fermentation process, while the other view claimed that the development was a purely chemical process. In the 1850s and 1860s, this argument was resolved when Louis Pasteur's work was presented to the scientific world. Pasteur was requested by the distillers of Lille, where the production of alcohol from beet sugar was a flourishing local industry, to regulate the problem caused by the lactic acid formation in alcohol production. On investigation of the fermentation end product under the microscope, Pasteur could witness the typical yeast cells. It was noted that there were a great number of smaller rod- and sphere-shaped cells. When Pasteur placed a small volume of this material in a sugar solution, vigorous lactic acid fermentation was observed along with the development of a grayish deposit in the solution that provided evidence of the presence of rod- and sphere-shaped cells. Consecutive transfers of these cells led to the manufacture of lactic acid during fermentation and a rise in the number of observed cells. Pasteur contended that the cells were a new "yeast" that precisely transformed sugar to lactic acid during its growth. It was years later when it was understood that the new "yeast" was bacteria. Using a similar technique, Pasteur studied a number of organisms and the process of fermentation. He was able to demonstrate that different fermentation products formed were always associated with particular types of microorganisms. This detection had additional importance. Just as the diverse microorganisms caused different fermentation product formation from sugar, different diseases were known to arise from dissimilar microorganisms. These microorganisms did not arise spontaneously as were once believed, but that individual microorganism was a resultant development from pre-existing cells of the same type. Another concept was introduced which provided the evidence that by destroying the microorganisms in food stuffs and beverages or by preventing their presence in sterile products, spoilage of food and beverages could be prevented. This perception is then directed to the heat treatment of food stuffs and beverages which is popularly known as "pasteurization" (Manchester 2007).

3.4 Fungi in the Drug Industry

The early part of the twenty-first century saw the inclusion of fungi in the industrial processing of more than 10 out of 20 most lucrative products used in medicine. Among these top ten products, two were anticholesterol statins, the antibiotic penicillin, and the immunosuppressant cyclosporin A. Each of these is found to have an economic turnover of more than $1 billion per annum.

3.4.1 Antibiotics

3.4.1.1 Penicillin

It is derived from the fungus, *Penicillium chrysogenum*, and was primarily used effectively to treat an infection triggered by a bacterium. Natural penicillins have numerous drawbacks. They are broken down in the stomach which contains acid, and thus cannot be taken orally. They act also only on gram-positive bacteria making them narrow-spectrum antibiotics.

3.4.1.2 Cephalosporins

The original fungus known to produce cephalosporin was *Cephalosporium*, hence the name. Basidiomycetes, particularly from tropical regions, are known to produce a variety of these compounds, for example, cefixime and ceftriaxone. The only largely beneficial antifungal agent from fungi is griseofulvin. The source of the same was *Penicillium griseofulvin*. Griseofulvin is fungistatic rather than fungicidal. It is used for the treatment of dermatophytes as topical application as it accumulates in the hair and skin (http://archive.bio.ed.ac.uk/jdeacon/microbes/penicill.htm).

3.4.2 Immune Suppressants

3.4.2.1 Cyclosporin A

It is a primary metabolite of several fungi, including *Trichoderma polysporum* and *Cylindrocarpon lucidum*. Cyclosporin A is a cyclic peptide comprising 11 hydrophobic amino acids. It acts by inhibition of lymphocytes. The inhibition of T cell proliferation results in the suppression of the activation process connected with invasion by foreign bodies.

Gliotoxins: This immune suppressant belongs to a class of compounds called epipolythiodioxopiperazines. This is produced by many fungi like *Aspergillus fumigatus* (Demain 2014).

3.4.2.2 Ergot Alkaloids

They act on the sympathetic nervous system leading to inhibition of noradrenaline and sclerotin, causing dilation of blood vessels. They also act on the smooth muscles of the uterus causing contractions and their early use usually induces abortion.

Alkaloids are now produced in culture by strains of *Claviceps fusiformis* and *Claviceps paspali* (Demain 2014).

3.4.2.3 Statins

Fungal species such as *Aspergillus terreus* and *A. phoma* are used for the production of statins. *Aspergillus terreus* is a soilborne fungus that produces a secondary metabolite called lovastatin. *Aspergillus phoma* produces squalestatin. Both statins have been used to decrease or eliminate low-density lipoproteins from blood vessels in humans. Statins have been attributed to attracting stem cells toward damaged tissues where the stem cells then appear to regenerate the damaged tissue. An extensive range of compounds with antibiotic activity is also identified and studied to explore their possibility for use in human benefits. The majority of them have been rejected for use in medicine for the reason that they either have unwanted side effects or show the instability of the active compound (Demain 2014).

3.5 Fungi in Food Processing

The use of fungi as a constituent in the food production process has become very common in modern days. Western countries are yet to accept these food products even today except few exceptions which are visibly prominent in their food habits. Food processing using fungi refers to a process where the production or manufacture of these food products requires the assistance or involvement of fungi. For example, in the production of baked bread, the yeast is used in preparing the dough to swell so that bread would come out light and fluffy. In absence of yeast, bread would be much denser and harder. Blue cheese is another such example. Asian cultures have a huge variety of such food, a few of which have developed to be extensively produced and consumed widely across many countries. This is probably for the reason that a greater number of Westerners have become more audacious in their dietary habits that have resulted in these types of food becoming more commonplace in our society. Predominantly this is true, in Hawaii, where there is a large Asian population and the mushrooms are excluded from such foods as they are the actual food product rather than the mushrooms being used to create other food products. A large number of fungi are integrated into their production using different processes (Martin and Coton 2017).

3.5.1 Cheese and Fungi

3.5.1.1 Moldy Cheeses

Though the sight of mold in food is a sign of contamination that should be discarded, there are some foods in which the existence of visible fungal mycelium is very much a part of the product. Among these are a few of the cheeses. Two of the most familiar examples are Camembert and Roquefort cheese, which are also branded as blue cheese. These cheeses are among the favorites among gourmets. These cheeses are

produced from two species of *Penicillium*, namely, *Penicillium camemberti* in Camembert cheese and *P. roqueforti* in Roquefort cheese. One of the most primitive known mass-produced food processing is the discovery of the cheese-making process which is assumed to have occurred around 4000 years ago. As was the case with any food that needed the accumulation of microorganisms, the detection of their occurrences in foods was invariably an accidental one. Because of the apparent magical conversion of milk into cheese, its origin has been masked in myths and folklores. One of the stories relates to an Arabian merchant carrying his milk with him in a pouch made from the stomach of a sheep. This is pertinent because a sheep's stomach contains rennet which is an enzyme that is essential in the cheese-making process. Rennet is the constituent that produces milk curdle and results from it into whey (liquid) and curd (solid). Over twilight, this procedure had happened and the merchant drank the whey and ate the curd. When he returned home, the merchant expressed his great discovery and how pleased he had been with his meal. In separating the liquid from the cheese, a product was described that had much of the similar qualities of milk and could be enjoyed as a food for a journey that would not spoil quickly and was also lighter than the original milk product (http://www.botany.hawaii.edu/faculty/wong/BOT135/Lect16.htm).

According to some folklores, it would be another 2000 years before molds and other microorganisms were supplemented to cheese for flavor. This development was assumed to have happened accidentally. After all, why would one deliberately place the mold on cheese? The basis of Roquefort cheese, according to Roquefort publicists, in an earlier period was that a young French shepherd boy took his sheep out to graze near the little village of Roquefort. Throughout this time an unexpected downpour happened that forced the boy to take shelter in a cave. The boy had to go out and round up the sheep; the moment the rain stopped, he took the sheep home. Nevertheless, he had forgotten his lunch. It would be more than a few weeks before he would return to that specific cave to find that his lunch was still there where he had left in his last visit. He could see the dried and crumbled bread; the cheese seemed to have veins of green growing all over. While it looked very bad, the boy was starving and took a bite from the discolored cheese and realized that it tasted better than any cheese he had tasted earlier. Thereafter, the boy ran down to the village, shouting, "a miracle, a miracle." This caused the people to gather around the boy and also taste the cheese, and ever since they started carrying cheese to the caves around Roquefort so that it could be converted into blue cheese. Those caves even today are still utilized for the production of Roquefort cheese. This is the story that is so often told that even school children, by the time they reach the second grade in Roquefort, know this story verbatim. Historically, no caves seem to be as well matched for the manufacture of Roquefort cheese as the one in which it was discovered first. *Penicillium roqueforti* is omnipresent and hence it was hard to continue a monopoly on this cheese in Roquefort. The people of Roquefort dreaded that other cheeses of low quality manufactured under their name would abolish the status of their Roquefort cheese. To avoid such an incident, the good people of Roquefort went to King Charles VI, who governed that only the cheese of Roquefort could be called Roquefort. Even though this would be revoked later, the use of Roquefort is still

limited by French guideline to avoid cheesemakers from misrepresenting the source of their cheese (https://myfoododyssey.com/2013/08/20/roquefort-cheese-mouldy-bread-comes-good/).

Nowadays, cheese which is produced using *Penicillium roqueforti* and not from the town of Roquefort is generically referred to as blue cheese from the blue-green mycelium of *Penicillium* growing through the cheese (Ropars et al. 2016).

3.5.2 Food Flavor and Color

The development of new colors for the food industry is challenging because colorants must essentially be compatible with food flavors, safety, and nutritional value and are associated with the price of the product. Furthermore, food colorants should preferably be natural rather than synthetic compounds. Microorganisms produce industrially beneficial natural colorants such as carotenoids and anthocyanins. Microbial food colorants can be produced on large scale at relatively small costs. Fungi are accountable for a variety of flavors of terpenes, menthol, and lactones. Fungi yield a variety of compounds that modify the color of food. For example, *Monascus purpureus* has been conventionally used for the production of red wine. Engineering processes using fungi are of great economic significance. The products are unique and typically there are no other economic ways for the production of these products (Ogbonna 2016).

3.6 Conclusion

There are several products produced on an industrial scale utilizing fungi as.

illustrated above in this chapter. Fungi play an important role in addressing main global challenges related to food and food products. Usage of fungal procedures and products can lead to improved sustainability and competent use of natural resources. Fungi are used in industrial fermentative processes involving the production of vitamins, pigments, lipids, glycolipids, polysaccharides, and polyhydric alcohols. Fungi bear antimicrobial activities and are used in biomineralization, as food with high protein contents, and even as biofertilizers. Fungi are enormously beneficial in the production of myco-proteins which act as plant growth promoters as well as disease suppressors. The importance of fungi at an industrial scale is enormous and well established leading to its extensive use around the world.

Conflicts of Interests Statement The authors declare no conflict of interest**.**

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4 Microbial Fermentation: Basic Fundamentals and Its Dynamic Prospect in Various Industrial Applications

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Abstract

The term "fermentation" refers to the anaerobic catabolism of sugars present in the extract of fruit or malted grains by yeast to produce carbon dioxide bubbles, which give a boiling appearance to the broth. Classically, unicellular eukaryotic fungus *Saccharomyces cerevisiae*, also referred to as "brewer's yeast," has been used to produce alcohol from malt or fruit extracts on an industrial scale. Thus, alcoholic fermentation using yeast strain is an example of microbial metabolite production. Similarly, several commercially important bioproducts such as microbial enzymes, biomass, recombinant products, and other platform chemicals are produced by microbial fermentation using a wide variety of substrates. Depending on the physical state of the nutrient medium, fermentation processes are broadly divided into submerged and solid-state fermentation. A typical fermentation process has the following components: (1) formulation of nutrient medium for developing the inoculum (producer microorganism) and to be used in the production fermenter; (2) sterilization of the medium, fermenters, and accessory equipment; (3) production of metabolically active inoculum in sufficient quantity which is free from any contaminating microbes; (4) inoculation and subsequent growth of the producer microorganism in the fermenter under optimum condition; (5) product recovery and purification; and (6) safe effluent disposal. Here, in this chapter, we intend to provide a comprehensive review on

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_4

the various fermentation processes, strategies for media formulation, and optimum growth of process microorganisms for industrial production of bioactive metabolites, recombinant proteins, and other added-value products.

Keywords

Fermentation · Aerobic · Chemostat · Lactic acid

Abbreviations

DO	Dissolved oxygen
DOE	Design of experiments
LAB	Lactic acid bacteria
OFAT	One-factor-at-a-time
PBD	Plackett-Burman design
RB	Rice bran
SmF	Submerged fermentation
SSF	Solid-state fermentation

4.1 Introduction

Fermentation is a process of generating energy where complex raw materials are converted into useful products. The definition of fermentation is different for different researchers. For some researchers, the term means any process for the production of a product by the mass culture of microorganisms, and for others, it is an energy-generating process in which organic compounds act as both electron donors and acceptors (Doelle 2014). In the fermentation process, the energy is produced by microbial enzymatic oxidation of any organic substrate such as carbohydrates, proteins, etc. The products of carbohydrate fermentation may be ethanol, lactic acid, citric acid, and butanoic acid, and fermentation like acetic acid and propanoic acid carbohydrate is not directly involved, but products of carbohydrate are used (Horton 2012). The basic raw materials used in the fermentation process act as carbon source, nitrogen source, salts, and cofactors for microorganisms. Polysaccharides are commonly used substrate for microbes used in fermentation processes, but sometimes oils and glycerol are also used as raw materials for fermentation (Glittenberg 2012). To produce maximum essential products during the fermentation process, factors such as temperature, pH, dissolved oxygen, and status of nutrients must be regulated and controlled. The most important factor is to minimize the risk of harmful contamination of microbe by carrying out under sterilized environments (Anderson 2009).

Microbial fermentation can be categorized into many criteria that may include the production of microbial biomass or microbial metabolites, enzymes, and

recombinant products (Doelle 2014). The production of different products during fermentation depends on the enzyme complex of cells and conditions of the environment of different microorganisms (Pandey 2003). But most importantly various biological functions of microbe like metabolism, reactions by enzymes, and biosynthesis result in production of fermentation products. Fermentation has diverse applications, and one of them is the improvement of organoleptic properties of food and enhancing of food safety by producing many metabolites that inhibit or kill food-borne pathogens.

4.2 Types of Fermentation Based on Substrate Used

4.2.1 Surface Fermentation

The fermentation process where the microorganisms used grow mainly on the surface of solid or liquid raw materials is known as surface fermentation. It can be easily controlled and implemented which are the main advantages of this fermentation, but its use in industries is limited because of its complexity. The aeration and agitation of media are the main basic operations of fermentation processes. The main edge of using this fermentation is needless of aeration or agitation of fermentation media. The products can be easily separated in this fermentation because microorganisms are not dispersed into the medium. Hence expenses and losses of product recovery are low. Surface fermentation has many advantages, but it also has several limitations like high investment cost on building, high personal expenses in countries with high wages, and long fermentation time (Pandey 2003).

4.2.2 Solid-State Fermentation

The fermentation technique in which microbial metabolites are produced on solid raw materials is known as solid-state fermentation (SSF). The main hallmark of solid-state fermentation is that raw materials contain enough moisture for the growth of the microorganism. The main benefits of using SSF are high product productivity, low energy consumption, low wastewater production, and relatively low moisture requirement for microbial growth and metabolism. SSF has various applications in the recently industrialized world which include bioremediation, production of microbial secondary metabolites, growth hormones for plants, organic acids, biofuel, and aromatic compounds (Pandey 1992; Agrawal and Verma 2020; Verma and Madamwar 2002).

4.2.3 Submerged Fermentation (SmF)

It is a type of bioprocess in which microbes are employed to break down the complex nutrient into value-added simple bioproducts in solution. This type of fermentation is

carried out in a closed vessel where the microbes are allowed to grow in liquid substrate like molasses, broth, etc. (Subramaniyam and Vimala 2012). In SmF, nutrients in the fermentation broth are consumed rapidly; hence it is very necessary to maintain the constant input of nutrients or replace the media with fresh media (Vidyalakshmi et al. 2009). In SmF, bacteria are the most preferred fermenting agent than any other microbes, as they grow better in high moisture conditions and exhibit high water activity (Babbar and Oberoi 2014). SmF is carried out in batch-fed fermentation and continuous fermentation. There are various factors that influence SmF such as substrate selection and fermentation agent. The selection of the right substrate is a prime matter of concern as the yield of fermentation highly relies on the nature of the substrate used. An appropriate modification of fermentation is necessary to get fruitful productivity from the particular substrate (Agrawal et al. 2019; Bhardwaj et al. 2019; Kumar et al. 2018). Substrates like soluble sugar, molasses, liquid media, sewage/waste, vegetable, and fruit juice are more frequently used (Vidyalakshmi et al. 2009). Microbes like bacterium are best suitable for this fermentation process. The SMF is mostly used for industrial purposes because of low cost, high yield, low contamination, and easy recovery of the product (Babbar and Oberoi 2014). The main limitations of SmF are high production cost, low productivity, and complexity of the medium (Fang et al. 2012). Most of the time, the submerged fermentation is used because of low expenditure, low operation time, and lesser space requirement and investments in labor, and on industrial scale sterilized conditions can be easily maintained due to simple process operations and easy to nurture property (Pandey 2003). However, SmF has various drawbacks compared to other fermentation processes which include higher funding for machinery, high consumption of electricity, high sensitivity to any irregularity in process parameters, susceptibility to microbial contaminations, etc. (Pandey 2003).

4.3 Types of Fermentation Based on the Availability of Oxygen

4.3.1 Aerobic Fermentation

Aerobic fermentation occurs at the beginning of the fermentation process and in the presence of oxygen. This is also referred to as “aerobic glycolysis” in living cells, wherein a glucose molecule is converted into two molecules of pyruvic acid through a sequential enzyme-catalyzed biochemical reaction consisting of ten steps. Following glycolysis, pyruvate is further catabolized into three molecules of carbon dioxide through the tricarboxylic acid (TCA) cycle in the cytosol and mitochondria of prokaryotes and eukaryotes, respectively. As compared to anaerobic fermentation, this process is shorter and more intense. Due to the low solubility of oxygen in water, dissolved oxygen (DO) is made available to individual microbial cells by aeration or agitation. Shake flask culture technique usually employs agitation to keep the culture at a high cell density by enhancing oxygen transfer rate in the fermentation broth to obtain higher yields of desired products (metabolites). Similarly, in bioreactors, mechanical agitation is provided by an agitator or impeller blades (such as in

continuous stirred-tank reactors) or in an airlift bioreactor, where aeration is ensured by gas purging for proper mixing of nutrients and biomass (Huang and Tang 2007).

4.3.2 Anaerobic Fermentation

Anaerobic fermentation is usually a slower process and occurs when oxygen in the fermentation broth is replaced with nitrogen, carbon dioxide, or any other by-product of the fermentation process (Huang and Tang 2007). In the absence of oxygen, pyruvic acid which is the final product of glycolysis can be directed toward two different routes depending on the type of cell. In brewer's or baker's yeast (*Saccharomyces cerevisiae*), the alcoholic fermentation pathway converts pyruvic acid into ethanol and carbon dioxide, whereas, in lactic acid bacteria (*Lactobacillus* spp.), lactate is produced by the lactic acid fermentation pathway. Certain obligate anaerobic microorganisms such as *Clostridium* spp. can produce butyric acid (short-chain fatty acid) which finds wide applications in chemical, food, and pharmaceutical industries (Xu and Jiang 2011). Several organic acids such as formate, propionate, butyrate, and acetate were produced from waste potato starch by anaerobic digestion using mixed microbial culture (Ayudthaya et al. 2018). Similarly, anaerobic fermentation has been considered as a feasible option for the generation of biogas and biomethane using microorganisms from various organic wastes including lignocellulosic biomass, animal wastes, municipal solid waste, and waste generated from food processing plants (da Silva et al. 2017; Kumar and Verma 2020a, 2021a).

Fermentation could be used in various industrial applications; some of them have been shown in Table 4.1.

4.4 Processes of Fermentation

The process of fermentation is dependent on the metabolic pathways of microorganisms involved in fermentation. Industrial fermentation is the regulated use of these metabolic pathways for the manufacture of products useful for people. Many industrially important products are manufactured using anaerobic fermentation. Some of the most important types and the kinetics involved are discussed in detail below:

4.4.1 Lactic Acid Fermentation

Lactic acid fermentation has been used for a long time for the preparation of fermented beverages and food all over the world. Many industries like dairy, cider, fermented vegetable, and meat rely on lactic acid fermentation (Taskila and Ojamo 2013). The overall relations involved in lactic acid fermentation are represented by Fig. 4.1.

Table 4.1 Microbial fermentation processes for the synthesis of various important industrial products

Substrate	Microorganisms	Type of fermentation (aerobic or anaerobic)	Product	Reference
Glucose, molasses	*Bacillus subtilis* and *bacillus licheniformis*	Aerobic fermentation	Poly-γ-glutamic acid (natural polymer)	Sirisansaneeyakul et al. 2017
Cane sugar or sugarcane molasses	*Rhodosporidium toruloides* NRRL Y-27012 and *R. Kratochvilovae* Y-43	Aerobic fermentation	Microbial lipids (single cell oil)	Boviatsi et al. 2020
Glucose	*Corynebacterium glutamicum*	Aerobic growth-arrested bioprocess	4-Hydroxybenzoic acid (aromatic compound)	Kitade et al. 2018
Waste activated sludge	Acidogenic microorganisms of the genus *Proteiniclasticum*, *Fusibacter*, *Macellibacteroides*, and *Petrimonas*	Anaerobic digestion	Volatile fatty acids	Huang et al. 2016
Glucose, Fe_3O_4 magnetic nanoparticles	*Enterobacter aerogenes* ZJU1, *Syntrophomonas*, and *Methanosarcina*	Anaerobic digestion/ dark fermentation	H_2, methane	Cheng et al. 2020
Cheese whey	*Lactobacillus acidophilus*	Anaerobic fermentation	Biogas, organic acids	Pandey et al. 2019
Cashew apple juice	*Saccharomyces cerevisiae* WUR 102 and *Hanseniaspora guilliermondii* CBS 2567	Anaerobic fermentation	Low alcoholic beverage with aroma compounds (β-phenylethanol and its acetate ester)	Gamero et al. 2019
Ovine (70%) and goat milk (30%)	Probiotic *lactobacillus paracasei* SP3 (isolated from kefir grains)	Anaerobic (fermentative lactic acid pathway)	Feta cheese	Mantzourani et al. 2018

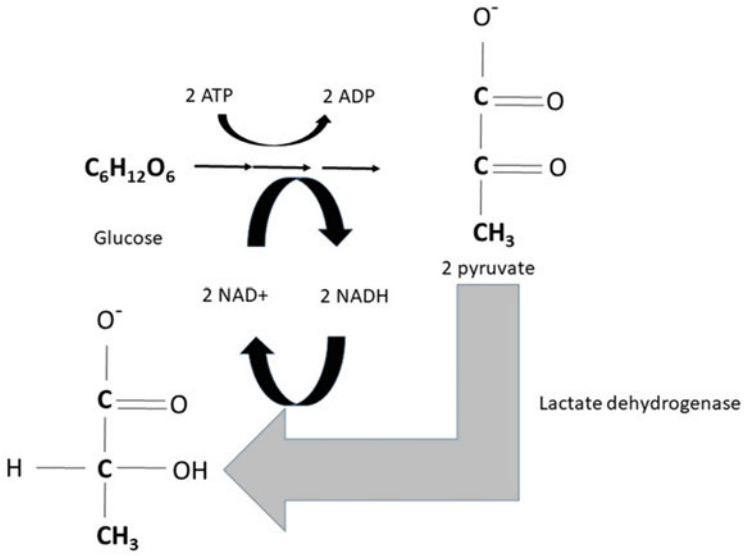

Fig. 4.1 Reactions involved in lactic acid fermentation

Lactic acid bacteria (LAB) play the most pivotal role in this type of fermentation. LAB is a group of bacteria that can utilize hexoses and produce lactic acid as the major product. There are three groups of LAB, namely, homofermentative, heterofermentative LAB, and bifidobacteria. Homofermentative LAB produces two molecules of lactic acid and ATP.

$$C_6H_{12}O_6 + 2ADP + 2Pi = 2C_3H_6O_3 + 2ATP \tag{4.1}$$

Heterofermentative LAB produces alcohol and carbon dioxide along with lactic acid.

$$C_6H_{12}O_6 + ADP + Pi = C_3H_6O_3 + C_2H_5OH + CO_2 + ATP \tag{4.2}$$

Bifidobacteria are responsible for the production of acetic acid as a by-product along with lactic acid.

$$C_6H_{12}O_6 + ADP + Pi = C_3H_6O_3 + C_2H_5OH + CO_2 + ATP \tag{4.3}$$

Since homofermentative LAB does not produce any other by-products, they are the most commonly used microorganisms for lactic acid fermentation. However, due to the ability of the heterofermentative LAB to utilize pentoses, they can be utilized for food preservation, mainly of plant origin (Zaunmüller et al. 2006). All three types of bacteria have extensive use as probiotics (Gomes and Malcata 1999).

4.4.2 Alcoholic Fermentation

Alcoholic fermentation involves the conversion of different sugars into basically ethyl alcohol and carbon dioxide by yeasts and some other organisms. The process starts with the breakdown of sugars into pyruvic acid, which is subsequently converted into acetaldehyde under anaerobic conditions. Acetaldehyde further releases two molecules of carbon dioxide and forms ethyl alcohol (Malakar et al. 2020). Figure 4.2 gives a brief idea about the reaction.

The most common ethanologenic microorganisms are yeasts which include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida* spp., *Kluyveromyces lactis*, *Pichia* spp., etc. Bacteria such as *Zymomonas mobilis*, *Clostridium sporogenes*, *Clostridium sordellii*, *Sarcina ventriculi*, and *Leuconostoc mesenteroides* are also involved in alcoholic fermentation (Binod et al. 2013; Kumar et al. 2020a, b; Kumar and Verma 2021b; Bhardwaj et al. 2020a, b).

Anaerobic Fermentation: Anaerobic fermentation occurs in certain yeast species such as *Saccharomyces*, *Schizosaccharomyces*, *Debaryomyces*, *Brettanomyces*, *Torulopsis*, Nematospora, etc. (De Deken 1966). The process of production of ethanol in presence of oxygen is known as Crabtree effect. Yeasts showing Crabtree effect can produce ethanol at high substrate concentration (Verduyn et al. 1984).

4.5 Kinetics During Fermentation

Microbial growth and product formations are interrelated events that depend upon many factors. The desired product may be some metabolic intermediates or the growing cells themselves and depending upon the nutrient requirements might differ [Clarke 2013]. Apart from nutritional requirements, other physicochemical parameters like pH, temperature, osmolarity, and oxygen availabilities also play an important role in fermentation. The discipline which deals with the optimization of

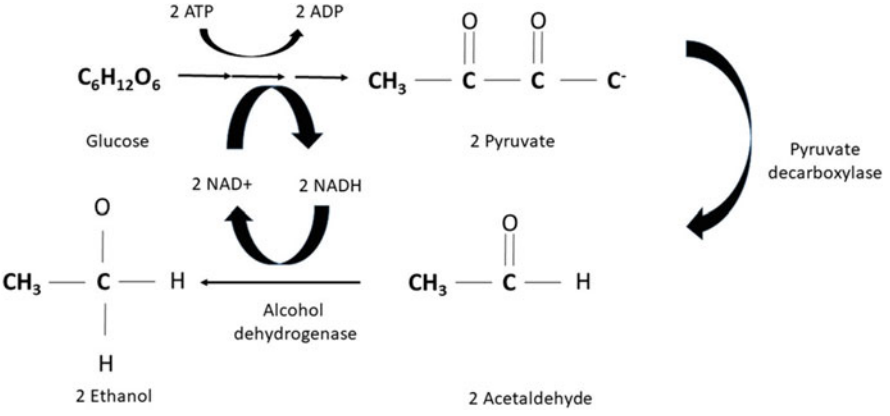

Fig. 4.2 Reactions involved in alcoholic fermentation

fermentation product yield through the utilization of different kinetic models of microbial growth is called bioprocess engineering. The product formation in a fermentation process is directly proportional to the rate of utilization of substrates. Therefore, in bioprocess engineering, different mathematical or kinetic models are developed to predict the microbial growth rate and the rate of product formation. The most important bioprocess strategies that are utilized for this purpose are batch cultivation, fed-batch cultivation, and continuous cultivation.

Batch cultivation: Batch cultures are designed for maximum product recovery during fermentation which minimizes the overall production cost [Yang et al. 2019]. It is the simplest culture technique that can be carried out on a laboratory scale under appropriate environmental conditions. The pioneering work on batch culture was done by Buchanan in 1918 and later by Monod in 1949. During batch culture, microbial growth can be classified into four phases: lag, log, stationary, and phase of decline. The lag phase defines that period of time after the time of inoculation where there is no visible sign of growth. During this phase, microbial cells adapt to the changing environment and start producing enzymes and other biomolecules required for the next phase, i.e., the log phase. The duration of the lag phase varies from organism to organism and also depends upon cultural and environmental parameters (Hill 2015). During the log phase, microbial cell division takes place and cell number increases exponentially. Exponential growth in bacteria is represented in terms of doubling time or generation time (G), in which the bacterial population doubles. Log phase cannot continue indefinitely and after a certain point of time due to the accumulation of metabolic products, cell lysis starts. When the rate of cell lysis equals the rate of cell division, the culture is termed stationary phase culture. During the stationary phase, cell culture product harvesting can be done. If the death rate exceeds the rate of cell division, then the culture is said to be in its death or decline phase.

Monod proposed a kinetic model for bacterial growth which states that bacterial growth rate is a function of limiting substrate concentration (Monod 1949).

$$\mu = \mu_{\max} \frac{[s]}{k_s + [s]} \tag{4.4}$$

where μ represents the growth rate, $\mu_{\max}$ is the specific growth rate of the microorganism, $[S]$ is the limiting substrate concentration, and k_s is the velocity constant.

The same model can also be applied for biodegradation as shown in Eq. (4.5) (Kovárová-Kovar and Egli 1998).

$$q = q_{\max} \frac{[s]}{k_s + [s]} \tag{4.5}$$

where q is the specific rate of substrate utilization (biodegradation).

Monod also coined the term yield coefficient ($Y_{x/s}$) and related to the specific rate of biomass growth (μ) and specific rate of utilization of substrate (q) by the following equation:

$$\mu = \frac{Y_{x/s}}{X} \times \frac{ds}{dt} \cong Y_{x/s} \times q \tag{4.6}$$

There are many modifications to the Monod equation, and the most common is the growth inhibition at high substrate concentration which is given by the equation below (Sokol 1986):

$$q = q_{\max} \frac{[s]}{k_s + [s] + {}^{S2}/_{K_i}} \tag{4.7}$$

where K_i is the inhibitory constant. This equation models when the substrate concentration is inhibitory for the growth of a particular microorganism.

4.6 Kinetics in Continuous Culture

A continuous culture or a chemostat is a culture that is operated with constant nutrition feed, and the parameters like the ratio between the substrate and the product and the environmental conditions are kept constant. Hence, the specific growth rate of an organism is kept constant and a steady state is maintained (Novick and Szilard 1950). In the bioreactor, at a steady state, the specific growth rate (μ) is found to be equal to the dilution rate (D). Dilution rate is the flow of the nutrient per unit time inside the bioreactor over the total volume of the bioreactor.

The mathematical model for a chemostat is represented by the formulae given below (Ziv et al. 2013):

$$\frac{dx}{dt} = \mu_{\max} \frac{S}{K_s + S} - Dx \tag{4.8}$$

$$\frac{ds}{dt} = DR - Ds - \frac{x}{y} \mu_{\max} \frac{S}{K_s + S} \tag{4.9}$$

where Eq. (4.4) denotes the change of cell density over time, assuming cell death is negligible. Eq. (4.5) describes the rate at which the limiting nutrient concentration changes with respect to the concentration of the inflowing media (R), its dilution by outflowing (s), maximum growth rate (μmax), cell density (x), and the yield constant (y).

4.6.1 Fed-Batch Culture

This type of culture can be regarded as a modification of batch culture since nutrients are supplemented after a certain period of fermentation. During batch fermentation, catabolic repression is observed due to the accumulation of products. The main aim of the fed-batch reactors is to minimize catabolic repression and enhance productivity (Özadali and Özilgen 1988). Moreover, the accumulation of biomass becomes

equal to the biomass generated and the exponential phase is maintained. For fed-batch reactors, Doran (2013) derived a formula as given below:

$$\frac{dx}{dt} = x(\mu - D) \tag{4.10}$$

where x is the biomass, μ is the specific growth rate, and D is the dilution rate.

In terms of yield, the following equation was derived:

$$\frac{ds}{dt} = D(S_i - S) - \left(\frac{\mu}{Y_{xs}} + \frac{qp}{Y_{ps}} + m_s\right)x \tag{4.11}$$

where Si is the inhibitory substrate concentration, Y_{xs} is true biomass yield, Y_{ps} is true product yield, qp is the specific rate of product formation, and m_s is the maintenance coefficient.

4.7 Different Media Formulation for Fermentation and Optimization of Media Components

As discussed earlier, the product yield in microbial fermentation depends upon many parameters, and those parameters have to be optimized for the highest yield. Some of the important parameters are discussed below.

4.7.1 Nutritional Requirements

Different media are used as growth media for microorganisms, but the basic components of most of the media include a carbon source, nitrogen source, and inorganic salts.

4.7.1.1 Carbon Source

The carbon source is the most important component of the media since different microorganisms yield energy from different sugars added to the media. The microbial biomass production and the yield of primary and secondary metabolites depend on the nature of the carbon source added (Marwick et al. 1999). Different microorganisms can utilize different sugars as carbon sources and the rate of utilization varies from microorganism to microorganism. There are also examples where a bacterium can prefer a particular carbon source over another when cultured in presence of both the sources (Monod 1949). Many carbon sources may act as interfering agents for the production of secondary metabolites such as antibiotics (Gallo and Katz 1972).

4.7.1.2 Nitrogen Source

Similar to carbon sources, the selection of nitrogen sources is also important for primary and secondary metabolite production during fermentation. There are many reports which show that use of certain amino acid repressed the production of certain metabolites (Singh et al. 2007). During wine fermentation, the production of hydrogen sulfide and ethyl carbamate was reported to be intensified by specific amino acids (Wu et al. 2018).

4.7.1.3 Inorganic Salts

Different types of inorganic salts are used in microbial culture media, and phosphate is one of the most important salts which actively participate in many vital processes of bacteria like the production of phospholipids (Singh et al. 2017). Production of many metabolites solely depends upon the concentration of phosphate ion, and above a certain concentration, the production is stopped (Sanchez and Demain 2008). Ammonium and phosphate ions were found to be promising for optimum ethanol yield, and magnesium was found to have a negative impact on alcoholic fermentation (Veljkovic et al. 1989).

4.7.2 Effect of Physical Parameters

Different physical parameters like temperature and pH play a very important role in the optimum recovery of products. Lipid production by *Fusarium solani* in batch fermentation was found to be optimum below temperature 35 °C in combination with other media components (Maia 2001). It was reported that biohydrogen production increases with temperature and decreases with pH (Xiao et al. 2013). The temperature of lipid metabolism strongly influences the odor components of wine. Fermentation of grape must be at the temperature as low as 13 °C to show better aroma components (Beltran et al. 2008).

From these reports, it is very clear that a slight change in a particular nutritional component may change the entire scenario of the fermentation process. Therefore, all the components are needed to be standardized for maximum production of a particular metabolite.

Different strategies of media optimization are utilized for the development of complete media. Optimization can be divided into two types: classical and modern (statistical) optimization (Singh et al. 2017).

Classical optimization involves a one-factor-at-a-time (OFAT) type of optimization. In OFAT optimization, at a particular time, only one factor is allowed to vary keeping other factors constant. Using OFAT, enhanced production of polysaccharides by *Stenotrophomonas daejeonensis* and *Pseudomonas geniculate* was achieved in which factors like fermentation time, carbon source, and nitrogen source were varied one at a time (Abou-Taleb and Galal 2018).

For advanced statistical methods, two main criteria are selected: finding the most significant factor and optimization of the component composition. Statistical methods or design of experiments (DOEs) suggests that changing more than one

component may be more efficient than OFAT (Fisher 1992). Moreover, DOE was found to involve less time and material to obtain similar results. Different experimental designs are available which provide a full factorial design for multiple components or factors involved in fermentation. R.L. Plackett and J.P. Burman designed a DOE known as Plackett-Burman design (PBD) (Mead et al. n.d.) which is a two-level design that is very important in differentiating the significant effects from the negligible ones. Plackett-Burman design is helpful for screening "n" number of variables using "n + 1" number of experiments (Reddy et al. 1999). Taguchi developed a method based on "orthogonal arrays" to overcome the shortcomings of PBD (Mori 2011). The Taguchi approach provides advantages such as shorter experimental time, improved quality of the product, and lesser product cost which is the primary aim for the process optimization of any fermentation method (Chanin et al. 1990).

After PBD, another model was developed by Box and Wilson (1951) which is popularly used in creating a second-order (quadratic) polynomial using the response surface methodology (RSM). An alternate to PBD is the Box-Behnken design which is independent of the quadratic model, which does not contain an embedded factorial design (Ferreira et al. 2007).

4.8 Cell Reactors Used in Fermentation

4.8.1 Immobilized Cell Reactor

The main requirement of modern bioreactor systems is biocatalyst (e.g., microorganisms, plant, or animal cells) that is immobilized into or onto a solid support matrix to prevent the cell washout, thus remarkably enhancing the concentration of biocatalyst while providing for maximum contact area with the substrate. The two most commonly used methods for cell immobilization are entrapment or encapsulation and adsorption. While the former method entraps cells into organic polymers such as polyurethane foam and absorbents like calcium alginate beads, the latter method adsorbs cells onto a solid support by physical or chemical interactions.

Immobilized cells have many different applications such as the production of pharmaceuticals and reagents, commodity chemicals, the fermentation of carbohydrates to ethanol, production of gaseous fuels such as methane and hydrogen from waste materials, production of macromolecules like enzyme, food, and beverages, and wastewater treatment (Clarke 2013).

4.8.2 Immobilized Enzyme Bioreactors

When immobilized enzymes are packed into columns and used in a flow system, they are known as immobilized enzyme reactors (IMERs). Usually, the separation of enzymes and products after reaction completion is difficult. However, the immobilized enzyme offers high catalytic efficiency, specificity, and also enhanced

tolerance to heat, pH, and organic solvents (Binod et al. 2013). Immobilized enzymes play an important role as biocatalysts in several industries like pharmaceuticals, food processing, and bioremediation purposes (Kumar and Verma 2020b; Agrawal and Verma 2020; Kumar et al. 2019). Since attachment of enzymes into or onto a support matrix (generally an inert and insoluble material) provides advantages improved stability and reusability as compared to free enzymes thereby allowing continuous use and product purification economically feasible at an industrial scale. The support matrix can be divided into two categories based on chemical compositions: inorganic and polymeric materials. The polymeric materials have sound mechanical properties and can be easily modified to fit specific requirements. Moreover, the specific activity and stability of immobilized enzymes can be improved by simply modifying the surface chemistry of the solid support. Enzyme-immobilized membrane bioreactors (EMBR) combine enzymatic catalysis with product separation. The selective membrane aims to separate the enzymes from the reaction products. The EMBR has been widely used in the chemical synthesis of antibiotics, anticancer drugs, several amino acids, vitamins, and optically pure enantiomer or even wastewater treatments and food processing (da Silva et al. 2017).

4.9 Application of Natural Substrates in Fermentation in Industrial Production of Bioactive Compounds

The metabolites comprising alkaloids, antibiotics, peptides, phenolic compounds, pigments, polysaccharides, etc. obtained from plants, algae, microorganisms, animal products, other seafood, etc., which demonstrate potential pharmacological applications, are known as bioactive compounds (Chye et al. 2018). The bioactive compounds have antimicrobial, antioxidant, antithrombotic, anti-inflammatory, and antiallergenic activities and, thus, are being used in curing chronic diseases, inhibition of carcinogenesis, anxiety, heart diseases, blood pressure, etc. to promote good health (Kris-Etherton et al. 2002; Kumar et al. 2020a, b). Due to the wide range of applications of bioactive compounds in food, and pharmaceutical industries, their extraction from natural sources has intensified using different strategies, viz., solid-liquid extraction (Tušek et al. 2016), ultrasound- and microwave-assisted extraction (Garcia-Vaquero et al. 2020), using high pressure (Alexandre et al. 2017), and supercritical fluids (Alvarez et al. 2019). Nowadays, the extraction of bioactive compounds from natural sources employing the process of fermentation is gaining attention due to its potent bioconversion property. A wide variety of natural substances have been converted to valuable products along with bioactive compounds using different processes of fermentation like solid-state fermentation and submerged fermentation (Sadh et al. 2018). Fermentation is regarded as an efficient method for the synthesis of bioactive compounds as it employs the machinery of microorganisms and microbial enzymes for the production of bioactive compounds through secondary metabolic synthetic pathways (Handa et al. 2019). Industrial production of bioactive compounds through fermentation has replaced the use of toxic solvents, hazardous chemicals, and other expensive physical methods

and, thus, serves as an eco-friendly approach to bioactive molecule synthesis (Magro et al. 2019). Agricultural residues, fermented foods, industrial effluents, vegetables, fruits, plants, cereals, and food by-products are commonly used substrates for industrial production of the bioactive compound through fermentation (Sadh et al. 2018).

Verardo et al. (2020) demonstrated that fermentation enhances the accumulation of bioactive molecules, viz., γ-aminobutyric acid (GABA), lycopene, and other phenolic compounds, and decreases the concentration of toxic substances like biogenic amines (BA). Sabater et al. (2020) described industrial production of bioactive metabolites by fermenting food wastes and by-products using lactic acid-producing bacteria, *Bacillus subtilis*, *Fusariumflocciferum*, *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., and many more. Fermentation-based succinic acid production utilizing *Saccharina latissima* as feedstock for a biorefinery approach serves as an eco-friendly alternative to petroleum-derived production of succinic acid and was demonstrated by Marinho et al. (2016). Wang et al. (2018) synthesized bioactive enzyme cellulase by submerged fungal fermentation of textile wastes using *Trichoderma reesei* ATCC 24449, an illustration of circular waste-based biorefinery. The enzyme has been used for the recovery of glucose and polyester residues from textile wastes as value-added products. Kombucha fermentation was employed for the synthesis of bioactive molecules from winery effluents using kombucha culture (*Acetobacter* sp., *Zygosaccharomyces* sp., *Saccharomyces*, *Torulopsis* sp.) (Vukmanović et al. 2020). Fermentation resulted in the increase of total phenolic content (TPC) and accumulation of other organic acids including acetic acid, oxalic acid, and a small amount of lactic acid in the beverage. The beverage showed pronounced antioxidant activity in comparison to other teas. Molinuevo-Salces et al. (2020) utilized a biorefinery approach for the production of biofuels by fermenting apple pomaces (AP) along with anaerobic co-digestion of manure.

The alcoholic fermentation study demonstrated an enhanced production of bioethanol and methane using *Kluyveromyces lactis*, *K. marxianus*, and *Lachancea thermotolerans* yeast strains and concluded that AP could be used as a potent candidate for biogas production. Olszewska-Widdrat et al. (2020) demonstrated the production of lactic acid from different renewable substrates by using *Bacillus coagulans* A534 through both batch and continuous fermentation experiments. Rice bran (RB), a by-product of rice cultivation, in general, and defatted rice bran (DRB) in particular have been used for the production of pure L-lactic acid without the addition of external supplements using *Bacillus coagulans* A107 isolate through batch fermentation (Alexandri et al. 2019). Acidogenic fermentation or anaerobic co-digestion of cheese whey and sewage sludge (used as inoculum) produced volatile fatty acids like lactic acid, acetic acid, and butyric acid (Iglesias-Iglesias et al. 2020). Agricultural wastes and by-products, forest wastes, sewage, household waste, industrial wastes, etc. could be employed for the production of various bioactive compounds beneficial for living beings. Thus, fermentation provides a cost-effective and alternative approach for the extraction of valuable bioactive molecules from natural resources which have a wide range of applications in day-

Table 4.2 Production of bioactive compounds from different biological sources through fermentation

Source/substrate	Microorganism	Bioactive compound	Fermentation type	Activity	Reference
Ecklonia cava (seaweed)	*Candida utilis*	Phlorotannin	Solid-state fermentation	Anti-inflammatory	Wijesinghe et al. 2013
Industrial wastes	*Streptomyces fradiae* NCIM 2418	Neomycin	Solid-state fermentation	Antimicrobial	Vastrad and Neelagund 2011
Black tea	Kombucha consortium	Organic acid and phenolic compounds	Solid-state fermentation	Antioxidant, anti-inflammatory, anticancer	Villarreal-Soto et al. 2019
Olive mill and winery wastes	*Aspergillus Niger* and *A. ibericus*	Xylanases, β-glucosidases, cellulases	Solid-state fermentation	Antioxidant	Filipe et al. 2020
Milk	*Lactococcus lactis*	Peptides	Solid-state fermentation	Antithrombotic and hypocholesterolemic	Rendon-Rosales et al. 2019
Opuntia ficus-indica and *Lantana camara*	*Saccharomyces cerevisiae*	Organic acid	Solid-state fermentation	Antioxidant	Tsegay 2020
Wheat straw	*Inonotus obliquus*	Phenolic compounds	Submerged fermentation	Antioxidant	Zhao et al. 2020
Celery seeds	*Bacillus subtilis*	Celery seed protein	Liquid state fermentation	Higher bile salts binding capacity	Chen et al. 2020

to-day life. Table 4.2 describes some bioactive compounds obtained from different biological sources employing potent microorganisms through different types of fermentation processes and their subsequent applications in food quality and human health.

4.10 Conclusions

In conclusion, fermentation is a process that modifies or breaks substances into simpler ones usually employing the metabolic activity of microorganisms including yeast and bacteria. Fermentation processes have been of utmost importance for human beings since time immemorial due to the wide variety of potential applications. The process of fermentation has evolved during the course of time with the development of technology and other engineering processes. Optimization of media and development of cell reactors has amplified the process of fermentation and the production of fermentation-driven products on a commercial scale. Large-scale fermentation of natural products employing microorganisms is a source of a wide variety of fermented foods and other bioactive compounds which could be used as potential functional foods and nutraceuticals. Fermentation-derived bioactive metabolites and other value-added products have been used as antimicrobial, anti-oxidant, anti-inflammatory, anticoagulant, and anticancer agents.

Declaration of Interest The authors declare that they do not have any conflict of interest.

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Fermenter Design

5

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Abstract

A fermenter (bioreactor) is a closed vessel with sufficient aeration, agitation, temperature, and pH regulation arrangements and a drain or overflow vent to extract the waste biomass along with its products from cultured microorganisms. The fermenter is intended for the production of biological products. The fermenter's design and mode of operation depend primarily on the production organism, the optimal working condition needed to create the target product, the product value, and the production scale. Fermenter is a consent to design criteria including sterilization ability; simple construction; easy calculation, monitoring, control techniques; scale-up; flexibility; long-term stability; upstream process compatibility, antifoaming steps. Fermentation is accomplished in several forms, including batch, fed-batch, and continuous fermentation process. The current chapter addresses the fermenter configuration, fermentation mechanism, fermenter types used in industries, heat and mass transfer in the fermenter, and the scaling up and regulation of the industrial fermentation process.

Keywords

Fermenter · Scale-up · Upstream · Heat transfer · Mass transfer

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_5

Abbreviations

ATP	Adenosine triphosphate
CIP	Clean in place
CSTR	Continuous stirred tank reactor
GFP	Green fluorescent protein
HDBR	High-density bioreactor
OTR	Oxygen transfer rate
PTFE	Polytetrafluoroethylene
RBBR	Rotating bed biofilm reactor
SIP	Sterilize in place
SSC	Set-point supervisory control

5.1 Introduction

During the First World War (1914–1918), Chaim Weizmann, alongside his team, formed a methodology for processing acetone by deep liquid fermentation using *Clostridium acetobutylicum*, primary to the subsequent usage of initial large-scale fermentation vessels. Contamination has frequently remained a solemn difficulty, predominantly in the initial portion of the large-scale development phase. At first, no appropriate vessels existed, and challenges remained inadequate with lid-fitted alcohol fermenters as steam sterilizers because atmospheric pressure might not be accomplished. Large cylindrical mild-steel vessels with hemispherical tops and bottoms sterilized under pressure with steam were the primarily built fermenters. The difficulties of aseptic media or inoculum additions persisted. As a result, steps were taken to design and improve piping, joints, and valves that could achieve and maintain sterile conditions if necessary. Only the minor seed vessels were mechanically stirred, and the vessel's contents were agitated continuously by the large capacities of gas produced during fermentation.

In the 1930s, Central Europe used the first real large-scale aerobic fermenters to manufacture compressed yeast (Becze and Liebmann 1944). These fermenters entailed large cylindrical air tanks at the base through perforated pipe systems. Motorized impellers replaced them, progressing the rate of mixing and air dispersion. This indeed led to the compressed-air necessities being decreased by a factor of 5. Baffles prevented vortex formation. Also, at this time, it was known that 10–20% of the overall production cost could be the cost for compressed air. In the early 1930s, water and steam were provided for sterilizing the aeration tubes. Before 1940, ethanol, glycerol, acetic acid, citric acid, other organic acids, enzymes, and sorbose were the other essential fermentation products besides baker's yeast. These procedures used extremely discriminating surroundings such as acidic or anaerobic conditions or the usage of an unusual substrate, resulting in comparatively negligible contamination compared to acetone fermentation or subsequent fermentation of

aerobic antibiotics. An essential factor in imposing the formation of sensibly constructed and purpose-built fermentation vessels was to practice submerged culture techniques for the production of penicillin, in which aseptic environments, good aeration, and agitation were critical. The Distillers Company's solvent plant in Bromborough was the only required aeration equipment to make it ideal for penicillin production (Hastings 1971). On 15 September 1943, the large-scale manufacturing of penicillin by deep fermentation, with steel fermenters of volume 54,000 dm^3, was initiated by Terre Haute in the United States of America (Callaham 1944).

5.2 Fermenter Systems

The fermenter, recognized as the heart of the fermentation process, endures numerous reactions and activities. Throughout the exploration of fermentation technology, tasks such as downstream, upstream, and midstream study are involved. Upstream processes involve selecting a microbial strain distinguished by synthesizing a particular commodity's desired commercial value in industrial fermentation. In contrast, downstream processing requires appropriate techniques and methods for recovery, purification, and characterization of the desired fermentation product in the different stages that accompany the fermentation phase. It has the primary objective of recovering the target product to the requirements (biological operation, purity) effectively, reproducibly, and safely while optimizing recovery yield and minimizing costs (Fig. 5.1) (Gudin and Chaumont 1991; Agrawal et al. 2019;

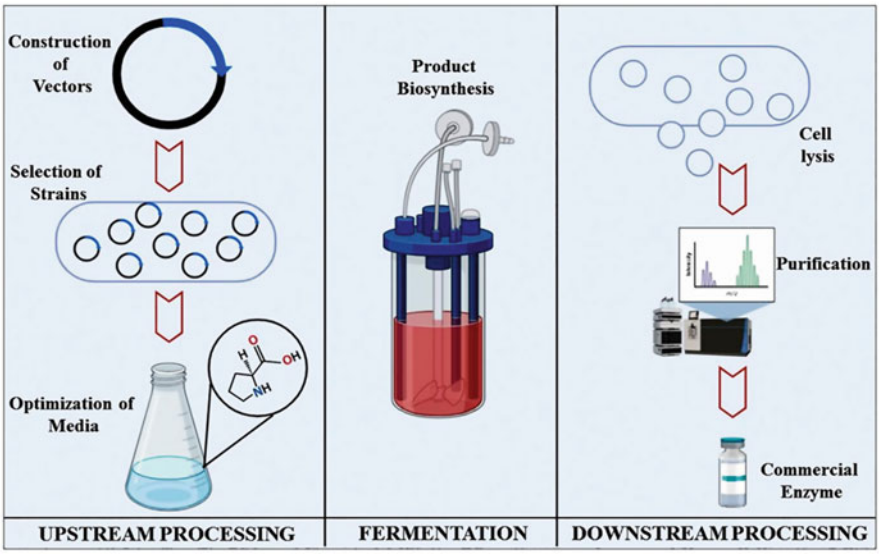

Fig. 5.1 Upstream, fermentation, and downstream strategies

Agrawal and Verma 2020; Bhardwaj et al. 2019; Kumar et al. 2018). The small distinction between the bioreactor and fermenter is that the fermenter is for microbial culture and the bioreactor is used for animal and plant cell mass culture (Gaikwad et al. 2018). Studies of fermentation technology comprise significant efforts from several fields of bioprocessing and chemical engineering, microbiology, biochemistry, genetics, and even physics and mathematics (Chandrashekhar and Rao 2010). The fermenter/bioreactor is a vessel that offers a biomechanical and biochemical environment that controls the movement of oxygen, nutrients to the cells, and metabolic products from the cell. The bioreactor vessels are constructed to use catalyst activity to achieve the anticipated chemical conversion (Sharma 2012). Bioreactors are also known as engineered devices designed to aid microorganisms, enzymes, biocatalysts, and plant and animal cells for the metabolic activity and optimal growth of organisms. For example, by regulating a culture temperature profile, some enzymes are denatured, organizing the cells' metabolic pathways over others. The fermenter or bioreactor is a cylindrical vessel with a hemispherical bottom and top shape and is mainly made of glass and stainless steel.

To some extent, bioreactors are distinct from conventional chemical reactors. The fermenter device is sufficient to control contamination when the organism is less stable and more susceptible than chemicals. To maximize shelf life and preserve acceptability, the development from natural art to commercial practice of these goods needs process enhancement. The consistency of the parameters of the product, equipment, and process control stayed simple. Generally, an aerobic system is supplied with sufficient substrate, salts, oxygen, suspended solids, and water. Products and by-products of gas production during the fermentation process need proper protection to be followed. Scale-up, controlling techniques, typical construction and calculation, sterilization, operational versatility, process control systems compatible with upstream and downstream processes, and antifoaming processes are considerations regarding fermenter design attributes specifications (Sharma 2012).

5.2.1 Functions of Fermenter

The primary purpose is to provide a regulated environment to obtain the desired product to grow microorganisms or animal cells. There are some criteria to be considered in designing and building a fermenter:

1. A vessel should work aseptically for some days with stable conditions and comply with the specifications of containment legislation for long-term service.
2. The metabolic requirements, adequate aeration and agitation of the microorganism's are important. The mixing does not, however, harm the organism.
3. Consumption of power should be as minimal as possible.
4. There should be a scheme for temperature control.
5. pH control is required.
6. Sampling facilities are needed.

7. Losses from evaporation at the plant.
8. In service, harvesting, cleaning, and repair, the vessel should be built to make minimum use of labor.
9. Preferably, the vessel should be appropriate for various processes, but this may be limited because of containment regulations.
10. The vessel design with even internal surfaces, wherever possible, using welds instead of flange joints.
11. The vessel's geometry in the pilot plant should be identical to smaller and larger vessels or installation to promote scale-up.
12. Use the cheapest material for better results.
13. Adequate service arrangements should be in effect for individual plants.

5.3 Parts of Fermenter

The fermenter design requires enhanced efficiency and authentication of the desired parameters with a high-quality product at a low cost. The fermenter's strategy and mode of operation are focused on the output of microorganisms, the product's cost, the condition required for the anticipated product development, and the scale of production. The successful fermenter has a beneficial effect on the biological reaction and avoids contamination from the outside. The essential considerations to be taken into account in fermenting capital expenditure and operating costs are adequate mixing, uniform shear rates, and monoseptic conditions to be preserved during the fermentation process. Culture aeration is accomplished by one of the succeeding approaches: raising ambient pressure or increasing the limited oxygen pressure and direct sparging, indirect and membrane aeration, or medium perfusion aeration (Eibl and Eibl 2008). The bioreactor enterprise must control pH, temperature, oxygen tension, aseptic feature, and shear stress. Several aspects of the biotechnological process require special care in the design of the bioreactor (Narendranathan 1986). Bioreactor conditions, such as pH, temperature, the velocity of agitation, dissolved oxygen level, development of foam, low gas rates, etc., are essential to regulate and closely track (Chen and Hu 2006). Figure 5.2 illustrates the parts of a basic laboratory-scale fermenter.

5.3.1 Construction Materials

Fermenters are constructed with materials that can resist constant steam sterilization and cleaning cycles. Substances that trace the fermentation medium and the broth must be nonreactive and nonabsorptive. For the development of fermenters with a capacity of up to about 30 L, glass is a good option. Smooth, nontoxic, corrosion-proof, and transparent are the advantages of glass. In common, glass fermenters are equipped with stainless steel head plates comprising several screw fittings since medium, inoculum, air, pH, and temperature sensors need entry ports. Most pilot and extensive fermenters are corrosion-resistant stainless steel, while mild steel with

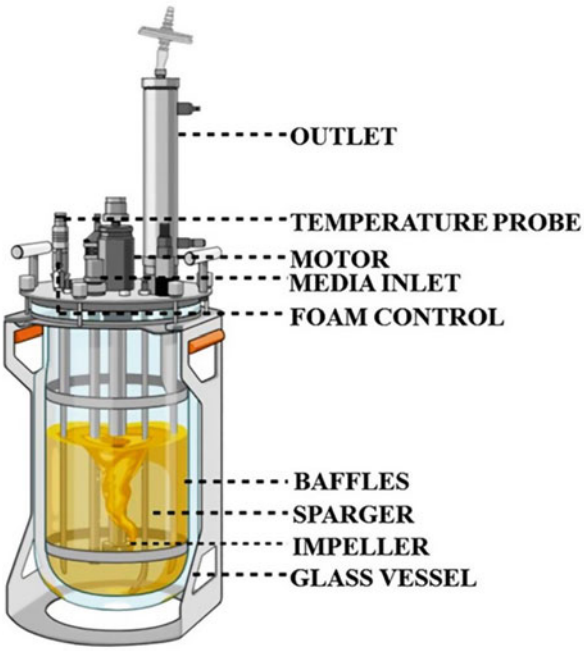

Fig. 5.2 Parts of a fermenter

stainless steel cladding can also be used. In fermentation areas that influence the culture, copper and copper-containing ingredients must be ducked as they are toxic to cells. Internal steel surfaces are refined to a superior mirror surface to enable cleaning and sterilization of the reactor; welds on the vessel's inner are ground flushed prior to polishing. Electropolishing is constantly chosen over mechanical polishing, which leaves minute ridges and trenches in the metal to accrue dirt and microorganisms.

5.3.2 Temperature Control

Generally, an appropriate temperature control provision in the design is required. Microbial action and mechanical agitation will produce heat, and if the heat produced by these two methods is not ideal for the precise production process, then heat will have to be applied to the system or removed from it. Usually, little heat is generated on a laboratory scale, and additional heat must be given by retaining the fermenter in a thermostatically operated bath or using inner heating coils or a heating jacket in which water circulates through a silicone heating jacket.

A dual silicone rubber mat by means of heating wires among the two mats contains a silicone jacket enfolded around the vessel and detained by Velcro strips. When a specific size is selected, the jacket's surface area becomes too small to extract heat from the fermentation. When this situation happens, it is essential to use internal coils and circulate cold water to accomplish the correct temperature. Diverse

forms of fermentation will affect the maximum vessel size that can be used for jackets alone. The fermenter's required cooling surface cannot be precisely defined because the cooling water temperature, the process of sterilization, the temperature of cultivation, the kind of microorganism, and the energy provided by stirring resolve significantly in different processes.

5.3.3 Aeration and Agitation

The primary aim of aeration is to afford adequate oxygen for metabolic requirements for microorganisms in submerged cultures. Simultaneously, agitation ensures that a constant holdup of microbial cells is attained in a homogeneous nutrient medium. Depending on the fermentation characteristics, the nature of the aeration-agitation device used in a specific fermenter differs. In fungal and actinomycete fermentations, mechanical agitation is generally necessary. Non-agitated fermentations are usually performed in vessels with a 5:1 height/diameter ratio. In such vessels, aeration remains adequate to create high turbulence, but in the processing of compressed air, a towering column of liquid requires more significant energy input. The structural components of the fermenter are:

(a) The agitator (impeller)
(b) Stirrer glands and bearings.
(c) Baffles.
(d) The aeration system (sparger)

5.3.3.1 The Agitator (Impeller)

The agitator is essential to attain a variety of mixing aims, e.g., bulk fluid and gas-phase mixing, air dispersion, oxygen transfer, heat transfer, stable particle suspension, and consistent atmosphere in the innards of the vessel. Agitators can be listed as disc turbines, vane discs, variable-pitch open turbines, and propellers. The disc turbine entails of a disc through a rectangular series around the circumference set in a vertical plane. A sequence of rectangular vanes is mounted vertically to the bottom of the vaned disc. Air from the sparger ranges the disc's bottom and moves to the vanes, where the air bubbles break down smaller bubbles. On the agitator shaft, the vanes of an adjustable pitch open turbine, and a marine propeller's blades are directly associated to the main. In this occurrence, earlier dispersion by the vanes or blades, the air bubbles do not originally trace any surface. The disc turbine in a fermenter is suitable because it might break down fast airstream deprived of flooding into air bubbles. When the vessel's bulk flow arrangement is typically related through the agitator arrangement, it is misplaced and exchanged by a centrally smooth air-broth plume up the middle of the vessel through a liquid flow as an annulus (Nienow 1990). The propeller is least effective at breaking down a stream of air bubbles. The flow produced is axial rather than radial and flooded lower than the disc turbine. The disc turbine is essential to thrust the sparged air hooked on the agitator tip zone where bubble breakup occurs. If the agitator rapidity is

sufficiently high, good gas dispersion will arise in low-viscosity broths (Smith 1985). Gas dispersion often occurs in high-viscosity broths from gas-filled cavities straggling behind the spinning blades but inadequate to safeguard adequate bulk mixing of all the vessel matters. The impeller tends to spin in a pocket of gas when the cavities are of full height, from which no actual dispersion takes place through the rest of the vessel. A dual impeller grouping implies suitable mixing and aeration in high-viscosity broths. The subordinate impeller performs like a gas disperser, and the upper impeller works predominantly as a device to aid the movement of the vessel's contents.

5.3.3.2 Stirrer Glands and Bearings

One of the most challenging problems in constructing a fermenter that can be worked aseptically for extensive periods is the adequate sealing of the top plate's stirrer shaft assembly. From the top, side (Richards 1968), or bottom of the vessel, the stirrer shaft gets into the vessel. The top entry is widely used, but if a different room is required on the top plate for entry ports, the bottom access is preferable, and the smaller shaft enables advanced stirrer speeds to be used by removing the issue of high-speed shaft whipping. The bottom entry stirrers are unacceptable as the bearings are submerged. Mechanical seals are used for bottom entry if sustained and replaced regularly at suggested intervals (Leaver and Hambleton 1992).

A porous bronze bearing is mounted in the top fermenter center and another in a yoke directly above it for a 13 mm shaft. The bearings are constrained into steel housings, secured into the yoke, and the fermenter's top into place. A skirt-like shield protects the lower bearing and housing with a 6.5 mm overhang that rotates with the shaft and stopped airborne pollutants from settling on the bearing and making into the fermenter. The stuffing box, the simple bush seal, the mechanical seal, and the magnetic drive are used as four specific seal assembly forms. As an alternative to stuffing boxes and packed glands, most modern fermenter stirrer mechanisms now integrate mechanical seals. Mechanical seals are costlier but are more robust and less likely to be a point of entry for pathogens or a moment of leakage for the organisms or items to be enclosed. Magnetic drives are used in assured animal cell culture vessels, which are also expensive.

5.3.3.3 Baffles

Four baffles are usually inserted into agitated vessels of all sizes to circumvent a vortex and upsurge aeration performance. Six or eight baffles are used in vessels of larger diameter. Baffles are metal strips approximately one-tenth of the diameter of the vessel and radially connected to the wall. With wider baffles, the agitation effect is only marginally increased but drops sharply with narrower baffles (Winkler 1990). A scouring action on the baffles and the fermenter walls around and behind the baffles minimizes microbial development. To increase the cooling power, extra cooling coils are connected to baffles of the fermenter without unduly influencing the geometry.

5.3.3.4 The Aeration System (Sparger)

A sparger is a device for introducing air into the liquid. Three specific sparger types are used and can be defined as the porous sparger, the perforated pipe orifice sparger, and the nozzle sparger (an open or partially closed pipe).

5.3.4 Feed Ports

Nutrient and acid/alkali additions to minor fermenters are usually made through silicone tubes that are separately autoclaved and pumped after an aseptic connection by a peristaltic pump. The nutrient reservoirs and associated piping in large fermenter units are typically an integrated component sterilized with the vessel. There can also, however, be ports that are in use intermittently.

5.3.5 Sensor Probes

Double "O"-ring seals offer a sterile closure for glass electrodes in fermenters. This method is also sufficient for levels 1 and 2, as long as the release of microorganisms is reduced and adequate disinfection procedures are available to deal with leakage (Werner 1992). Probes are built-in with triple "G"-ring seals at containment levels 2 and 3/4 (Leaver and Hambleton 1992). While double "G"-ring seals are denoted with steam tracing, they are not naturally considered achievable. As a way of coping with probe failure instead of using a retractable electrode housing throughout the fermentation cycle, pre-inserted backup probes are advised because of the chance of broth leakage (Sharma 2012).

5.3.6 Foam Control

It is essential to reduce foaming during any fermentation. The chances of filters getting wet when foaming becomes excessive, resulting in pollution. There is also the risk of siphoning, leading to losing some or all parts of the fermenter's content. Antifoams can cause aeration or downstream processing issues in some circumstances. The foam is guided over fixed turbine blades in a separator in certain fermenters, and the liquid fraction is reverted to the fermenter.

5.3.7 Valves and Steam Traps

Valves and ancillary devices connected to fermenters are used in several ways to regulate the flow of liquids and gases. The valves are required to:

1. Be easy as regards switching them *on*/*off* that are either completely open or fully closed.

2. Provide flow rates with coarse control.
3. Be very precisely calibrated to regulate flow rates.
4. Be safe so that liquids or gases flow in one direction only.

5.3.8 The Achievement and Maintenance of Aseptic Conditions

It is essential to sterilize and hold a fermenter and its contents sterile in a full growth cycle. The containment requirements depend on the size of the fermentation vessel. To achieve and maintain aseptic conditions during fermentation, the following operations are performed according to the specifications:

1. The fermenter's sterilization.
2. Sterilization of the inlet air and exhaust gas.
3. Agitation and aeration.
4. The addition of nutrients, inoculum, and others with supplements.
5. Sampling.
6. Controlling foam.
7. Monitoring and control of various parameters.

When conniving a fermenter to act as a contained device at level 1, B2, or higher containment levels, the following points must be considered:

1. All vessels comprising live species must be sterile vent filters and appropriate for steam sterilization.
2. Vessel exhaust gases can pass through sterile filters.
3. At the lower stages of containment, flange joint seals should be fitted with a single "O"-ring. Double "O"-rings or double "O"-rings plus a steam barrier are desired on flange joints on vessels for containment levels 3 and B3/4.
4. Seals for sensor probes, inoculum, sampling, medium addition, acid, alkali, and antifoam entry ports must be given.
5. A double-acting mechanical seal with steam or condensate between the seals can be used to seal rotating shafts into a bolted device.
6. A steam barrier must be recognized in all fixed piping prominent to the "contained" vessels during service.
7. Adequate pressure relief conveniences must be provided.

5.3.8.1 Sterilization of a Fermenter

The medium may be sterilized both in the vessel and separately before being applied aseptically. To avoid the increase of large quantities of condensate, the temperature of the sterilized medium should be increased before injecting live steam. Steam may be injected into the fermenter coils or jacket to accomplish this. Since any entry point to and exit from the fermenter can be a source of pollution, steam must be added through all entry and exit points except the air outlet, which should be left open.

To confirm that steam enters all sections of the machinery and is not blocked by siphons or pockets of condensate or mash, all pipes should be installed as simply as conceivable and grade into drainage points. A steam trap should be installed at each drainage point in the pipework. Welded joints should be used wherever possible for long-term aseptic service, even if parts need to be cut out and re-welded during maintenance and repair.

5.4 Properties of an Ideal Fermenter

The fermenter is a device used to convert low-value materials into higher-value products through microorganisms' controlled growth. Industrial production of highly valued brewed products, such as wine and beer, or other fermented products such as citric acid, acetic acid, etc. (Zhong 2010) would require a carefully choreographed sequence of events, which can be controlled by the various parts of the fermenter as described in the previous section. Working of a fermenter in ideal condition is essential for maximizing the profit from the industry. Some of the ideal properties of running a fermenter are shown below.

5.4.1 The Ability to Provide Contained Conditions

An ideal fermenter should be able to provide an aseptic condition for long periods. If the fermenter is not a closed system (except for components we inject deliberately), there can cause many complications such as the growth of unwanted microorganisms, which can decrease the overall product's efficiency. It can also produce unwanted products and our desired product that can hinder the desired product's ideal use. Such products being introduced into the system can make the downstream processing costlier. Microbial contaminations are known to cause considerable losses to companies since the whole batch needs to be discarded. Hence, providing a contained environment is top-notch for the fermenter's ideal working (Yang 2011).

5.4.2 The Ability to Provide an Ideal Working Environment

Most microorganisms have unique needs to work with maximum efficiency; these needs can be temperature, pH, and other chemicals. An ideal fermenter will need ports for the insertion of probes for temperature and pH. Systems have been developed with computer programing to monitor these parameters and adjust the same to fit the requirement. A heating coil, water jacket, or steam jacket is installed in a fermenter to control the temperature. Predetermined concentrations of acid or alkali are fed into the fermenter to maintain the ideal pH. Other chemical agents or enzymes are introduced according to the reaction's need, as they can be inserted into the reactor at selected times. Thus, an ideal reactor should sense and control the

above parameters without compromising the vessel's sterility (Yamuna Rani and Ramachandra Rao 1999).

5.4.3 The Construction Material

The construction material should be corrosion resistant, preventing the accidental insertion of trace metals into the system. The material should be able to withstand repeated sterilization. Usually, the vessel is a pressure vessel. For volumes less than 10 L, glass can be used, while vessels with larger capacity are made using various grades of stainless steel (302, 304, 316, 318). The material is usually polished to a mirror finish for ease of cleaning and preventing any dust and other components. The joints are welded and are smoothened out to prevent any sharp corners.

5.4.4 The Shape of the Fermenter

The fermenter is designed to withstand high pressure. The ideal shape is a cylinder with an arched bottom. The walls and bottom are made curved to withstand higher pressure and ease of cleaning. It also helps in the excellent mixing of the contents in the system. Aspect ratio is the ratio between the height of the reactor to its diameter, which should be kept between 1 and 3 for a continuous stirred tank reactor, whereas, for a tower reactor, the value can go up to 10.

$$\text{Aspect ratio} : \frac{H_t}{D_t}$$

where:

H_t is the height of the tank
D_t is the diameter of the tank

5.4.5 The Dimensions of Fermenter Parts

The various parts of the fermenter play a vital role in the system's stability and unhindered working. Standards have been established on ratios of various parts of the reactor such as impeller and baffles. The ratio of the diameter of the impeller to the diameter of the tank should be kept in between 1/3 and ½, the ratio of the diameter of the baffles to the diameter of the tank should be 0.08–0.1, the ratio of impeller blade height to diameter of the impeller should be 0.2, the ratio of impeller blade width to the diameter of the impeller should be 0.25, and the ratio of the distance between the middle of impeller blade to impeller blade height should be 1. These values are established in international standards for a stirred tank fermenter (Bates et al. 1963).

5.4.6 Aeration and the Impeller Velocity

The aeration rate should be fixed depending on the fermenter and organism substrates causing the fermentation (Rajavathsavai et al. 2014). If enough rate of aeration is not provided, it can prevent cell proliferation. If the rate of aeration is higher than required, it can lead to unnecessary foaming and other side reactions, which will decrease the reaction's efficiency. The impeller's velocity varies widely depending on the type of cells (animal cells or microbial organisms), reactor's size, the impeller (Rushton disc turbine, marine propeller, hydrofoil), nature of the product, etc. Ideally, the impeller's velocity is kept stable so that it does not harm the cells by shearing or destroying the end product.

5.5 Types of Industrial Fermenters/Bioreactors

Fermenters are suitable for producing a wide variety of products, from products as simple as ethanol using *Saccharomyces cerevisiae* to products as complicated as vaccines, interferons, and antibodies. A fermenter's design depends on the source of cells, the product, the production scale, etc. Depending on the requirements, various types of reactors are designed. Some of the different bioreactors are continuous stirred tank reactor, batch fermenter, tower fermenter, gas lift fermenter, deep jet fermenter, fluidized bed bioreactor, airlift bioreactor, bubble column bioreactor, photobioreactor, wave bioreactor, membrane bioreactor, and sparged tank bioreactor (Table 5.1) (Ali et al. 2018).

5.5.1 Continuous Stirred Tank Reactor

Continuous stirred tank reactor (CSTR) is the most utilized bioreactor. Almost 70% of the industrial bulk production using fermentation is performed using this type of bioreactor. In a CSTR, the media, cells, and products are continuously mixed with an impeller's help (Fig. 5.3b). This would mean that the composition of the media present in the reactor will be the same as the output media, making the CSTR ideal for research studies and calculations. The CSTR can be operated in the principles of chemostat and turbidostat. The chemostat involves using flow rates of the reactor to maintain a chemical equilibrium in the system, which is ideal for the fermenting microbes to produce maximum yield. Turbidostat is maintaining constant turbidity in the vessel. The turbidity measures the number of microorganisms present in the reactor (Wang and Zhong 2007; Ali et al. 2018). Stirred type bioreactors are discussed in detail in the next section.

Table 5.1 Types of bioreactors and their applications

S. no.	Type of reactor	Application	Organism	References
1.	CSTR	Brewing industry	Yeast	Yamauchi et al. (1995)
		Bioremediation of industrial effluents	Consortium of organisms	Gargouri et al. (2011)
		Biogas production	Methanogens	Jürgensen et al. (2018)
		Anaerobic waste treatment	Consortium of organisms	McCarty and Smith (1986)
2.	Gas lift fermenter	Growth of filamentous organisms	*Aspergillus oryzae*	Barker and Worgan (1981)
		Contaminant elimination	*Pseudomonas cepacia*	Ensley and Kurisko (1994)
		In vitro meat production (conceptual)	Animal tissue culturing	Li et al. (2020)
		Syngas fermentation		Asimakopoulos et al. (2018)
		Production of single-cell protein	*Cephalosporium eichhorniae*	Varavinit et al. (1996)
3.	Deep jet fermenter	Production of single-cell protein	Yeast	Bajpai (2017)
		Production of biopolymers	*Methylocystis* sp.	Listewnik et al. (2007)
4.	Packed bed fermenter	Removal of phenol	*Streptomyces psammoticus*	Niladevi and Prema (2008)
		Production of organic acids and hydrogen	*Enterobacter*, *bacillus*, and *clostridium*	Leite et al. (2008)
		Autotrophic denitrification	*Thiobacillus denitrificans*	Claus and Kutzner (1985)
		Clarification of juices	Immobilized pectinase	de Oliveira et al. (2018)
		Fermentation of beer	Yeast	Yamauchi et al. (1995)
5.	Fluidized bed fermenter	Wastewater treatment	Consortium of organisms	Bello et al. (2017)
		Development of starter cultures for difficult applications (metal working fluid treatment)	Consortium of organisms	Vyrides et al. (2019)
		Denitrification of water	*Micrococcus denitrificans*	Kurt et al. (1987)
6.	Photobioreactor	Production of secondary metabolites	*Scenedesmus ovalternus*	Koller et al. (2017)
		Production of single-cell protein	Purple phototrophic bacteria	Hülsen et al. (2020)

(continued)

Table 5.1 (continued)

S. no.	Type of reactor	Application	Organism	References
		Microalgae as shading for windows	*Scenedesmus obliquus*	Pagliolico et al. (2017)
		Wastewater treatment	Consortium of organisms	Ashok et al. (2019)
7.	Wave bioreactor	Recombinant protein expression (animal cells)	*Spodoptera frugiperda* 9 cells, BTI-TN-5B1-4 cells, HEK 293 cells, etc.	Ghasemi et al. (2019), Baldi et al. (2007)
		Recombinant protein expression (microorganisms)	*Escherichia coli*	Glazyrina et al. (2010)
		Tissue engineering	Various animal cell lines	Selden and Fuller (2018)
8.	Membrane bioreactor	Syngas fermentation	Depends on the product	Asimakopoulos et al. (2018)
		Wastewater treatment	Consortium of microorganisms	Yamamoto et al. (1989)
9.	Sparged tank bioreactor	Syngas fermentation	*Clostridium carboxidivorans* and *Clostridium ragsdalei*	Liu et al. (2019)
10.	High-density bioreactor	Production of biodiesel	*Chlorella protothecoides*	Xiong et al. (2008)
		Production of biopharmaceuticals	Depends on the product	Syed et al. (2021)
		Cell-based therapies	Red blood cells, induced pluripotent stem cells, mesenchymal stem cells, etc.	Stephenson and Grayson (2018)
11.	Microbioreactor	High-throughput bioprocessing	Depends on the study	Fink et al. (2021)

5.5.2 Batch Fermenter

A batch fermenter is a system where all the components required for the cell growth and production of products are added initially before starting the process and are run until the time of harvest. It is a closed vessel system. Batch fermentation is seldom performed industrially for biological products since the product in bulk quantities would cause a change in various parameters, which needs to be adjusted as required. This process is used for processes like crystallization, dissolution of multiple components, polymerization, etc. Another property of the batch fermenter is that it is performed in discreet batches. After completing each process, the product is harvested, and the process has to start again from scratch. This type of fermenter is used for high-value, low quantity yield products. A significant advantage of this type

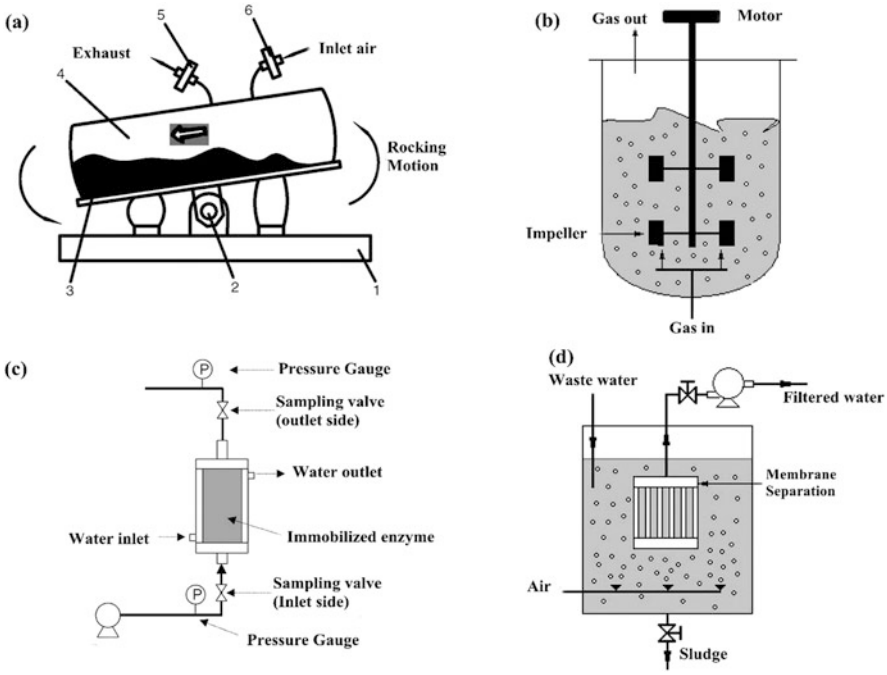

Fig. 5.3 Types of bioreactors. (**a**) Wave bioreactor (1, base; 2, pivot; 3, cell culture media in bag; 4, inflated plastic bag; 5, exhaust vent filter; 6, inlet air filter). (Adapted with permission from Zhong 2011). (**b**) CSTR. (Adapted with permission from Wang and Zhong 2007). (**c**) Packed bed reactor. (Adapted with permission from Watanabe et al. 2005). (**d**) Membrane bioreactor. (Adapted with permission from Wang and Zhong 2007)

of fermenter is that in case of contamination, unlike a continuous process, only a single batch of significantly less quantity is lost (Cardello and San 1988; Yeh et al. 2006).

5.5.3 Tower Fermenter

In simple terms, the tower fermenter is a tube with a tapering bottom with facilities to control various parameters like temperature and aeration. Tower fermenters are used mainly for continuous fermentation. The overall aspect ratio for the tower fermenter is 10, implying that the reactor's height is significantly higher compared to its diameter. The tower fermenter usually has a sparger at the bottom of the tower from which air is pumped in resulting in a nonmechanical stirring and ample aeration. This type of reactor is used for brewing beer. Owing to its high diameter, the tower fermenter can be used to create a gradient (yeast and wort gradient in the case of beer brewing). In beer production, the raw materials float toward the surface (Jones et al. 1984). This type of reactor usually has a perforated baffle sitting parallel

to the ground level, unlike the CSTR as it is the aeration that causes the mixing in the tank.

5.5.4 Gas Lift Fermenter

A gas lift fermenter is a vessel that has a central inner tube through which compressed gas is pumped. The air pumped will cause the mixing of the components in the media and bring about a loop circulation. Since it is using air for mixing, it is a non-mechanically stirred reactor. Heat transfer is also a result of the air circulated. The airlift fermenter is almost similar to the gas lift fermenter and bubble column fermenter. As compared to the bubble column fermenter, the airlift fermenter has a central tube that helps in proper mixing and circulation (Fig. 5.4a). An airlift reactor has two zones, the riser and the comer. The riser is the column where the air is pumped. There is no air pumped in the comer, which will result in circulation through the riser and comer. An adequate flow of air maintains the circulation. There are two types of airlift reactors, internal loop airlift reactor and external loop airlift reactor. The internal loop airlift reactor has a single central draft tube, which creates the circulating media loop that the external loop airlift reactor has separate channels for circulation. The advantage of using an airlift is it has very low shear stress making it ideal for animal cells. Due to the height of the reactor, there is an increased pressure toward the bottom, helping in easier mass transfer (Burkert et al. 2005).

5.5.5 Deep Jet Fermenter

A high-powered pump is useful in circulating the medium in a deep jet fermenter. An injector nozzle pumps in a jet of medium with compressed air. The gas forms giant bubbles that are removed from the port present on the top. The media then passes through the ejector nozzle, which degasses the media. The cooled aerated medium is finally injected into an air entrainer above the reactor (Jafari et al. 2012).

5.5.6 Packed Bed Reactor

Packed bed reactor/fixed bed reactors are reactors with packed (immobile) components such as enzymes or biocatalysts in a tubular or cylindrical arrangement (Fig. 5.3c). In general, the media will be pumped from the bottom of a packed column, and the effluent is taken out through the top simultaneously. Aeration can be provided from the bottom of the reactor, and the air outlet is placed at the top of the vessel. A packed bed reactor is used in wastewater treatment as the microbe forms a biofilm on the packed material (usually a biocatalyst), enhancing the process. A significant advantage of the fixed bed reactor is that the conversion rate per unit mass is higher since it has biocatalysts. Separation of catalyst from the product can easily

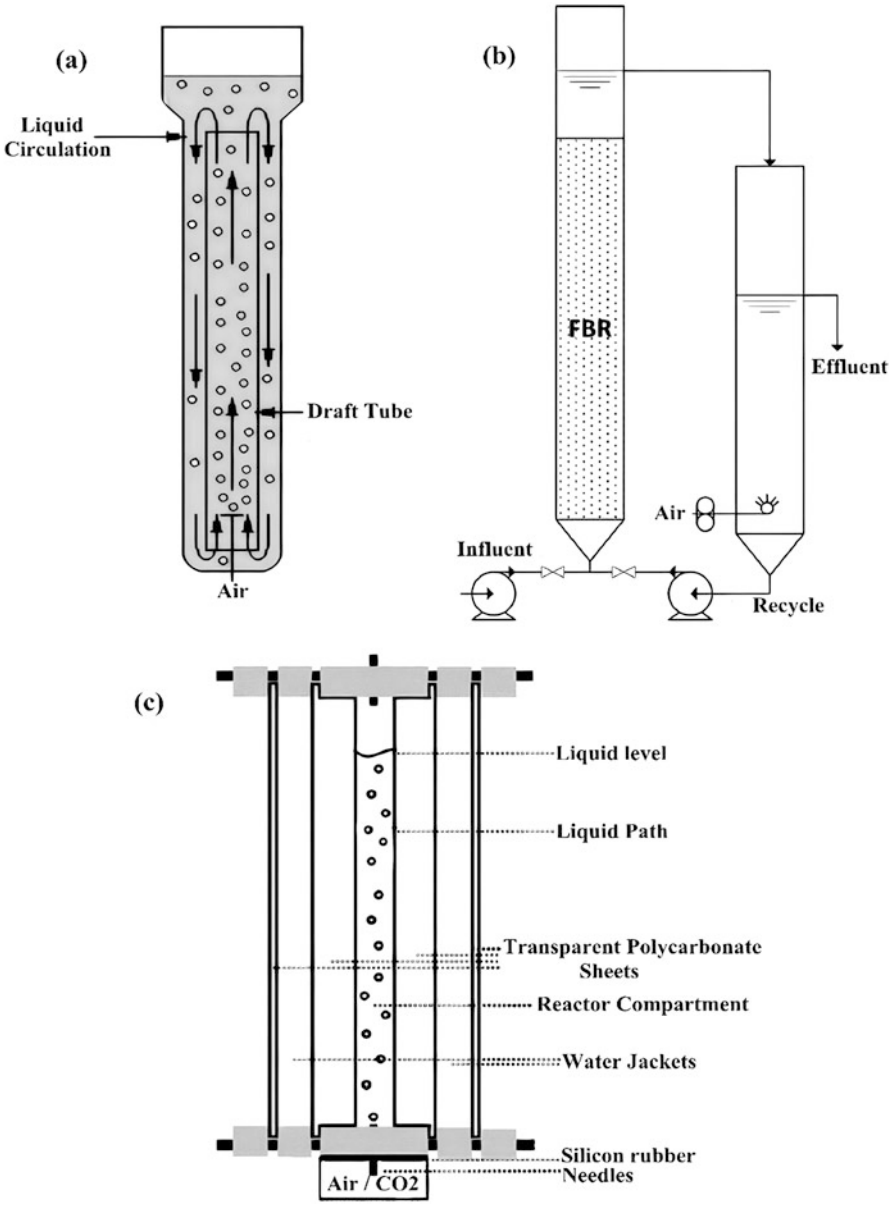

Fig. 5.4 Types of bioreactors. (**a**) Airlift bioreactor. (Adapted with permission from Wang and Zhong 2007). (**b**) Fluidized bed reactor. (Adapted with permission from Özkaya et al. 2019). (**c**) Photobioreactor. (Adapted with permission from Singh and Sharma 2012)

be achieved in these types of reactors. The packed bed reactor also provides the possibility of operation at higher pressures and temperatures (Shojaosadati and Babaeipour 2002; Watanabe et al. 2005; Zhong 2011).

5.5.7 Fluidized Bed Reactor

In a fluidized bed reactor, unlike the packed bed reactor, the materials are distributed in a fluid by its flow, i.e., the biocatalysts are suspended in the reactor due to the fluid flow rate acting themselves as fluid (Fig. 5.4b) (González et al. 2001). Like the packed bed reactor, the catalysts are held on a porous plate, which helps fluid pass through the material. At a lower fluid flow rate, the force imparted on the materials is not sufficient to lift the particles, and this system will act similar to a packed bed reactor. As the flow rate increases, the particles tend to lift up, and a stage is achieved where the particles' weight is suspended in the fluid and balances out due to the fluid flow. This process is referred to as incipient fluidization. After the incipient fluidization, as the fluid flow rate is increased further, the particles become fluidized and behave similar to a fluid, hence the name, fluidized bed reactor. Aeration can also be provided along with the fluid flow; however, the particle distribution is no longer uniform in the presence of gases. The fluidized bed reactor can be classified as a tower reactor as the aspect ratio is similar. These types of reactor has better temperature distribution and has significantly high contact with the particles, hence increases in the product yield (Özkaya et al. 2019). The reactor is used for the production of various chemicals like vinyl chloride, polypropylene, etc. It is also used in the brewing industry for the production of beer (Krishnan et al. 1999).

5.5.8 Photobioreactor

Photobioreactors are utilized to grow organisms that require light sources for their survival (phototrophic microorganisms). The reactor provides an artificial environment, cyanobacteria, mosses, algae, and plants by providing light apart from other nutrients. Photobioreactors can be classified as open type and closed type. The open-type photobioreactors are either natural open ponds or artificially created ponds with controlled conditions to grow the organisms. A closed photobioreactor is a closed laboratory system that provides all the nutrients and light with a highly reduced risk of contamination (Fig. 5.4c). Depending on the configuration, closed photobioreactors are divided into six categories: tubular glass photobioreactor, Christmas tree photobioreactor, plate photobioreactor, horizontal plate photobioreactor, foil photobioreactor, and porous substrate bioreactor. Interestingly a closed photobioreactor provides higher cell growth (Hoekema et al. 2002; Singh and Sharma 2012; Fu et al. 2019).

5.5.9 Wave Bioreactors

Wave bioreactors are comparably newer technology used for culturing both animal and plant cells. The bioreactor system is simple and cost-effective. The bioreactor can be made with polymers yet providing all the utilities of a regular stainless steel bioreactor. The polymer bag (bioreactor) is placed on a rocking platform. The platform's rocking motion creates waves that provide very little shear stress without compromising on the mixing (Fig. 5.3a). The wave action also prevents the accumulation of cells. A pre-sterilized bag is an advantage in a polymer bioreactor, and hence further sterilization is not required. The wave bioreactor also shows relatively less foaming due to its less vigorous wave action (Zhong 2011).

5.5.10 Membrane Bioreactor

Membrane bioreactors are mainly used in biological wastewater treatment. The membrane bioreactors can retain the microorganisms and other catalysts in the process tank while pushing out the treated product (Fig. 5.3d). In a water treatment process, the contaminated water comes in contact with the microbes inside the process tank, and only the clean water leaves out. The sludge along with the microbes remains in the process tank, which can be later removed. The membrane is usually made using polyamide, polysulfonate, and cellulose acetate. This bioreactor is employed for the formation of alcohols and acids (Wang and Zhong 2007; Chang et al. 2019).

5.5.11 Sparged Tank Bioreactor

Sparged tank bioreactors introduce air into a metal vessel from the bottom through a porous plate. This air non-mechanically agitates the system and also takes away the heat in the system. The gas bubbles get dispersed with the help of a baffle arranged horizontally. Since it is agitated by air, the system does not require an agitator and has comparatively less shearing stress. This type of reactor uses significantly low power since it has limited moving parts.

5.5.12 High-Density Bioreactor

A high-density bioreactor (HDBR) is a novel reactor design used to treat sewage wastewater with microorganisms' help. After the secondary wastewater treatment, the mixed liquor goes to a clarifier where the sludge settles down in the conventional activated sludge process. The practical and complete removal in a separate step is essential. The use of HDBR will not require this additional step. The HDBR system is a modified fluidized bed reactor in which the rate of the recycled stream is less with aeration. This will maintain the biomass zone in the reactor without disruption. The

reactor in itself is similar to a fluidized bed reactor with an inlet port on the bottom of the reactor, pumping in calculated quantities of influent with aeration, and an outlet port situated on the top to remove the feed which is partially recirculated into the inlet port (Sales and Shieh 2006). The HDBR is used for the high-density cultivation of algae to produce biofuels (Price et al. 2015).

5.5.13 Microbioreactors

Microbioreactors are a novel idea that lets us collect precise data using tiny quantities of media and microorganisms. Microbioreactors are scaled-down CSTRs and shake flask cultures. This type of bioreactor helps in screening for many numbers of microorganisms in a very cost-effective manner. A microbioreactor is especially helpful in animal cell cultures where the media and other components are significantly costlier than the microbial growth medium. These reactors can be used for growth profiling, running parallel cultures, standardization of fermentation parameters, media screening, toxicity screening, and phenotyping. The above parameters can be studied using fluorescence, pH, dissolved oxygen, spectroscopy, etc. Quantities of less than 1 mL are only required for all the above testing. After studying the microbioreactor parameters, the test can be carried out on a lab-scale quickly, followed by scale-up to pilot plant and industrial-scale cultivation (Ravindran et al. 2019). The bioreactor is a commercial microbioreactor manufacturer providing bioreactors for testing for multiple parameters (Toeroek et al. 2015).

5.5.14 Rotating Bed Biofilm Reactor

Rotating bed biofilm reactor (RBBR) was invented by Prof. Hallvard Ødegaard in the late 1980s (Ødegaard et al. 1994). It is a reactor with an aerated tank with a cylindrical carrier partially immersed in the growth medium. The cylindrical carrier is capable of rotation around an axis. The carrier forms a surface for the microorganisms to form a biofilm. The rotating carrier provides alternate exposure to media and air. They are mainly used for wastewater treatment. An advantage of RBBR is that it provides significantly higher areas for biofilms to form. It is also highly cost-effective and is easily scalable. It can also be used for the production of algae, in turn helping in biofuel production and for the production of products like ethanol (Shen et al. 2017).

5.6 Stirred Tank Reactors

Continuous stirred tank reactors (CSTR) are vessels made from glass or stainless steel with a motor-driven shaft with impellers that rotate to cause uniform mixing of components in the vessel supporting fermentation and thus producing high yields of value-added products. The vessel will also have ports for various purposes such as

sampling, acid inlet, alkali inlet, temperature, pH sensing, etc. The CSTR is the most employed fermenter for research purposes. This fermenter can be easily scaled up, and the mixture inside the reactor is assumed to be uniform, thus making the calculations more comfortable.

5.6.1 Geometry of CSTR

A CSTR is a pressure vessel capable of continuous sterilization and decontamination. It is a cylindrical vessel with an outward curved base. There will be no sharp joints inside the reactor; this will prevent the accumulation of any components in these sharp joints. The curved base helps in the uniform mixing of components. The aspect ratio of CSTR is kept between 1 and 3. The motor-driven shaft is usually fixed in the center of the reactor horizontally, with impellers radially outwards (depending on the type of impeller). The vessel is made using glass or stainless steel. Glass is used to construct vessels below 10 L, while stainless steel was used to construct reactors above 10 L. The outer and inner surfaces are usually polished to a mirror finish for easy cleaning and to prevent any accumulation. The vessel material and inside components are corrosion-resistant air.

5.6.2 Impeller

As the name suggests, the impeller is an essential component in a CSTR. It helps in the mixing of the components in the vessel. There are many types of impellers such as blade paddles, spiral propeller blades, ribbon blades, anchor blades, screws with draft tubes, etc. Selection of the impeller blade is crucial as each type of blade's properties are different, making it unique to different requirements. Blade paddle or paddle agitator or Rushton turbine is the most commonly used impeller blade. There is a two-blade and four-blade configuration. This blade is apt for slow operations. Paddles at high speeds can cause heavy shear stress on the cells, thus decreasing the efficiency. The axial flow hydrofoil impeller is a way to reduce shear rates. In this impeller, the liquid is either drawn up or down with lower energy requirements. This blade can thus be used in shear-sensitive processes involving animal cell culture. Another example of an axial flow impeller is the marine propeller (Ameur 2015; Gu et al. 2017).

The impellers need to be positioned ideally to achieve maximum oxygen transfer rate. If the impellers are too close to each other, the mixing quality is decreased drastically. If the impellers are placed too far apart, the uniformity of the overall solution is affected. One to 1.5 impeller diameter is the spacing that gives ideal mixing in most cases.

The selection of the impeller blades depends on the fluid's rheology inside the reactor. If the broth is too viscous, a helical ribbon is useful. Also, it is observed that the use of a Rushton turbine in non-Newtonian broth is seen to increase the viscosity of the broth near the central shaft, thus impeding proper mixing (Sossa-Echeverria

and Taghipour 2015). To solve such types of issues, the impeller should be custom-designed for the broth being used. Novel impellers such as Mixco A-315 have shown to give homogeneous mixing with almost the same power requirement as the Rushton turbine.

Hence the Impeller should be so selected to maximize air dispersion and oxygen transfer with ideal fluid-gas mixing. The blade type and rotation speed should not cause any shear stress on the organisms cultivated. The blade dimensions should provide homogeneous mixing and avoid any stagnant zones in the vessel. It should use less power for the same oxygen transfer rate it provides. A proper impeller with a precise rotation should be selected based on the parameters mentioned above.

5.6.3 Modelling of Ideal CSTR

A CSTR is useful for most of the research calculations, and in practical industrial applications, it is still a much-preferred system. It has several advantages like homogeneous mixing of contents in the vessel, fair heat distribution, good yield, etc. The following assumptions are made to model the design equation of an ideal CSTR (Liu 2017):

1. The mixing is entirely homogeneous, and, hence a sample taken from practically anywhere in the bioreactor will have the same composition.
2. The fermenter is in a steady state, i.e., there is no accumulation of contents in the reactor.
   $\left(\frac{\mathrm{dNA}}{\mathrm{d}t} = 0\right)$ where NA is the number of moles of species A.
3. The boundaries of the vessel are closed, and there is no mass transfer between the boundaries.
4. It is assumed that there is a constant fluid density.
5. There is a constant temperature.
6. The reaction is considered to be irreversible.
7. All the reactants are converted to products by the reaction.

Net accumulation

$$A = \mathrm{Ain} - \mathrm{Aout} + \mathrm{Ageneration}$$

$$\frac{\mathrm{dNA}}{\mathrm{d}t} = \mathrm{FAo} - \mathrm{FA} + V * \mathrm{VA} * \mathrm{rA}$$

where:

FAo is the molar flow rate of the inlet of species A
FA is the molar flow rate of outlet of species A
VA is the stoichiometric coefficient
rA is the reaction rate

$$0 = \mathrm{FAo} - \mathrm{FA} + V * \mathrm{rA} * \mathrm{VA}$$

Assuming steady state, VA = -1
Thus, $0 = \mathrm{FAo} - \mathrm{FA} - V * \mathrm{rA}$
We know that

$$\mathrm{FAo} = Q * \mathrm{CAo} \tag{5.1}$$

$$\mathrm{FA} = Q * \mathrm{CA} \tag{5.2}$$

where:

CAo is the concentration of inlet species A
CA is the concentration of outlet species A
Q is the fluid flow rate

$$0 = Q * \mathrm{CAo} - Q * \mathrm{CA} - V * \mathrm{rA}$$

$$\mathrm{rA} = \frac{Q}{V}(\mathrm{CAo} - \mathrm{CA})$$

$$\mathrm{rA} = \frac{1}{T}(\mathrm{CAo} - \mathrm{CA}) \tag{5.3}$$

where T is the theoretical residence time $\left(T = \frac{V}{Q}\right)$.

Residence time is the total amount of time wherein a quantity of compound will be retained in a bioreactor.

From Eqs. (5.1) and ((5.2),

$$\mathrm{FAo} = Q * \mathrm{CAo}$$

$$\mathrm{FA} = Q * \mathrm{CA}$$

$$\frac{V}{Q} = \frac{\mathrm{CAo} * \mathrm{XA}}{-\mathrm{rA}}$$

$$T = \frac{\mathrm{CAo} * \mathrm{XA}}{-\mathrm{rA}}$$

From Eq. ((5.3), the design equations from zeroth order, first order, second order, and nth order reactions can be calculated.

The size of the reactor (volume) can be shown using the Levenspiel plot, which is represented by $\frac{\mathrm{FAo}}{-\mathrm{rA}}$ on the X-axis and conversion in the Y-axis.

The reaction rate is most significant at the start of the reaction, which reduces in practicality.

5.6.4 Gas Delivery System

A gas delivery system or aeration system consists of a device called a sparger. It is used to introduce air into a bioreactor vessel. A perforated ring-type sparger is the most used sparger in a CSTR since it provides the most uniform air distribution. Aerobic culture requires significantly high air inputs; for a 100 m^3 reactor, almost 100 m^3 air is required to be pumped per minute. Continuous stirring in a CSTR provides ample air distribution in the system (Karimi et al. 2013). All the air that goes into the system needs to be sterilized. Details of the same are given in the sections below.

5.7 Strategies for Gases, Nutrient Solution, and Media Sterilization for Industrial Fermentation

Sterilization is the process of elimination of all viable organisms from a system. This process is of utmost importance to lab scale, pilot scale, and industrial fermenters. This failure would lead to the competitive growth of both our organism of interest and the invading species, which would mean that a significantly lower amount of nutrients will be available for the target organism, which will reduce the system's overall productivity. The contaminant organism can also produce unwanted products into the system leading to contamination of the product. These unwanted products can also make the downstream processing much more complicated, decreasing the firm's revenue. The contaminants may all ruin the whole process calculations for the systems, i.e., the contaminant organisms may have a completely different morphology (such as mycelia or more rigid cell wall) or can have a different growth curve. It may produce more heat, which will render the cooling system ineffective, thus killing the target organism. Another factor that influences the need for contamination is if the process is batch or continuous process. Contamination in a batch process is timely resolvable compared to a continuous process. In a continuous process, the contaminant organism will outgrow the target organism if not monitored closely. The loss of revenue by the loss of a continuous process is significantly higher than the batch processes. Hence, the need for sterilization is very high in any industry or research facility involving the use of fermentation (Stanbury et al. 2013).

To achieve a sterile environment, all the bioreactor components and vessels are needed to be sterilized. The sterilization strategies for the different components are different. For example, a vessel's sterilization strategy cannot be used to sterilize gases that enter the system. Similarly, a standard sterilization method is not useful for the sterilization of all media types; for example, we cannot sterilize the media for microbial culture and animal cell culture in a similar strategy. Thus, sterilization strategies for various components in an industrial fermenter are included in this chapter.

5.7.1 Strategy for Medium Sterilization

In theory, media sterilization can be performed by methods such as radiation, chemical treatment, filtration, ultrasonic treatment, and heat. But considering the pros and cons, heat sterilization is the most widely preferred method of sterilization for microbial cell cultures; on the other hand, filtration is the most sought-after method for media sterilization in animal cell culture since the media is heat-labile. In heat sterilization, sterilization using steam is the most preferred method mainly because of its high latent heat (Sternfeld and Paulus 1993).

Sterilization using steam can be much more comfortable in a laboratory-scale fermenter (less than 10 L). The whole system can be disassembled and can be taken to an autoclave where almost all the components are sterilized. This is not practical for a pilot-scale plant or an industrial-scale plant that will have huge parts that cannot be moved; hence clean-in-place (CIP) and sterilization-in-place (SIP) approach are used. In this approach, the cleaning and sterilization are performed at the place where the fermenter is installed (Pettigrew et al. 2015).

The sterilization-in-place approach is a widely used system in which the heat for sterilization is brought to the vessel, usually in the form of steam. These two approaches, either a steam jacket to heat the whole system or the steam, are pumped into the vessel and are often preferred. This strategy used depends on the process required. Sterilization with steam is usually carried out at 120 °C at 2 bar pressure, with a holding time of 60–70 min. The kinetics for sterilization is calculated according to the organism used and the product used. The sterilization time and temperature regime are pre-calculated.

The sterilization can be performed either as a batch process or a continuous process. Batch sterilization is preferred for laboratory and pilot-scale plants (Singh et al. 1989), while industries prefer continuous sterilization. This is mainly because in huge plants (100,000 L), it will be difficult to heat the media batch-wise. The core temperature will be different as compared to the borders. Robust impellers also will need to be employed for uniform heat distribution.

In comparison, the motors running the impellers will need less power, hence decreasing the industry's running cost. Studies have shown that 60–80% less water and steam are utilized for continuous sterilization. Continuous sterilization also helps in lowering turnaround time, implying more productivity (Pfeifer and Vojnovich 1952).

Continuous sterilization plate heat exchangers were used initially, but because there were immense chances of gasket failures (thus mixing sterile and non-sterile streams of fluids) and blocking of the heat exchangers due to particulates in the media, the system was replaced by spiral heat exchangers. This solved the problem of gasket failures and the two streams were separated by steel. The flow inside the spiral heat exchangers is self-cleaning, hence reducing the chances of blockage. A major disadvantage of the heat exchanger is the temperature regimes need to be controlled accurately. This is much easier in batch sterilization (since it can be controlled manually). Sophisticated computer programs have been designed to take over the control of these heat regimes. The computer-controlled continuous

sterilization is so accurate that the technique can be used to sterilize heat-labile media components with controlled heating and cooling regimes (Deindoerfer and Humphrey 1959).

As mentioned before, most of the sterilization is performed using steam (heat sterilization). Heat-labile nutrient feeds used in animal cell culture cannot be sterilized using this method as it decreases the nutritional availability in the media. The preferred method for sterilizing heat-labile media without any particulate matter is filtration (Coté 1999). The filtration is usually described by the following mechanisms: inertial impaction, diffusion, electrostatic attraction, and interception. In inertial impaction, the suspended particles' momentum causes them to collide with the filter membrane, while the fluid passes on through the membrane. Diffusion is caused by Brownian motion. The suspended particles, owing to their Brownian motion, tend to get stuck in the membrane. Charged particles get attracted by oppositely charged surfaces, such as filtration membranes. Interception is how the filter membrane will stop the suspended particles if the pore size is smaller than the particle itself (Tien and Ramarao 2011). In most of the filters, the pore size will be smaller than the particles' size to be filtered. These filters are arranged to form a sheet of cartridge accommodated in the housing. On a laboratory scale, a standard disc or capsule filter can be used for filtration. This can be achieved using a syringe to provide the necessary pressure. Industrial filtration requires more complicated filtration cartridges for this purpose. Zeta Plus™ Encapsulated System is a scalable depth filter that can be used for media sterilization. Most of the industries use membrane filters in cartridges with a pore size of 0.2 μm for filtration. The disadvantage of using membranes with such small sizes is that it can get easily clogged; hence, usually a prefilter is equipped in an industrial fermenter. A prefilter is generally a filter with a much bigger pore size to remove any coarse particles suspended in the solution.

5.7.2 Strategies of Gas Sterilization

Aerobic fermentation needs significant quantities of air, which is injected into the reactor through a sparger. A fermenter requires one volume of air per volume of medium per minute. This implies that vast quantities of air need to be pumped in to maintain the culture. This air also needs to be sterilized to maintain a contamination-free environment inside a bioreactor. The most commonly used method for sterilization of gases is filtration. As mentioned previously, the filtration method employed for gas sterilization is similar to the methods employed in media sterilization. The air is passed through a fixed pore membrane filter. The membranes are arranged to form a cartridge which is housed in plastic housings (for laboratory scale and pilot scale) or stainless steel housings (for industrial-scale bioreactors). The most commonly used filtration material is polytetrafluoroethylene (PTFE) (Wikol et al. 2008). The PTFE in itself forms a complicated mesh that reduces the pore size of the whole membrane; apart from this, it is also arranged in loops inside a cartridge to provide a complicated path for the gas to travel, thus filtering it. Another advantage of using a

PTFE membrane is that it is hydrophobic, thus repelling water from the membrane. In gas filtration, as in media filtration, there is a requirement of a prefilter to remove significantly larger debris. The prefilter is usually made of glass microfiber, polypropylene, etc.

The strategy usually followed by an industry in the sterilization of fermenter is by pumping steam into the reactor once the media is loaded. This would sterilize both the media and the vessel. But if the fermenter needs to be sterilized separately, the vessel may be heated using steam in heating jackets or electrically heated coils. Steam directly pumped into the reactor can also be used for vessel sterilization. The steam pressure is held at 15 psi at 121 °C for 20 min to sterilize the vessel. Once the sterilization is complete, sterilized air should only be pumped inside the bioreactor, and the bioreactor should always be kept at positive pressure. If this step is not performed carefully, air from surroundings may creep into the system due to the vacuum created because of the exit of steam and compromise the whole bioreactor's integrity.

5.8 Industrial Fermentation Processes

Industrial fermentation methods start with selecting suitable microorganisms and conditions, such as careful nutrient concentration adjustment. The by-product is diverse: alcohol, glycerol, and carbon dioxide from yeast fermentation of various sugars; butyl alcohol, acetone, lactic acid, monosodium glutamate, and diverse bacterial ACEs; and citric acid, gluconic acid, and small quantities of antibiotics, and vitamin B12 and riboflavin.

Five important commercial fermentation groups exist:

(a) Those that generate as a result microbial cells (or biomass)
(b) The manufacturers of microbial enzymes
(c) Those which generate microbial metabolites
(d) Those producing recombinants
(e) Those who change the transformation phase of a compound added to the fermentation

5.8.1 Microbial Biomass

Commercial expansion of microbial biomass is split up into two main processes: yeast production for baking and microbial cells' production for human and animal use (single-cell protein). Since the beginning of the 1900s, baker's yeast has been developed on a wide scale, and in Germany, in the course of the First World War, yeast has been produced as human food. Nevertheless, until the 1960s that microbial biomass was extended as a food protein source. These developments were focused on feedstock hydrocarbons, which could not compete against other high protein animal feeds (Sharp 1989). However, the decline of animal biomass feed

fermentations was composed by establishing the method for manufacturing fungal biomass.

5.8.2 Microbial Enzymes

Commercially plant, animal, and microbial source enzymes are developed. However, microbial enzymes have the enormous advantage that stable fermentation techniques can be produced in great numbers. Infinitely simpler than in a plant or animal system is the enhancement of a microbial system's productivity. Also, the introduction of recombinant DNA technology allowed for synthesis by microorganisms having animal origin enzymes. Development in microorganisms is controlled, and these controls can be generated over several phases of the growth curve to increase productivity. The products formed during the log growth process are essential for cell growth and include amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates, etc. These goods are called the basic metabolism products, and a step (log equivalent or exponential) in which the metabolism is formed is referred to as the trophophase.

The industrial microbiologist is responsible for modifying the wild organism and creating cultural conditions to increase such compounds' productivity. Some microbial cultures synthesize in the deceleration and stationary phases compounds that have not been generated during the trophophase and do not seem to be apparent to cells' metabolism. These are considered secondary compounds and their phase (stationary phase equivalent) as an idiophase. It is necessary to note that secondary metabolism is a feature of the slow-growing and the nongrowing cells in continuous cultivation at low growth rates. Suppose the microorganisms evolve in their natural environments at relatively low growth rates; it is tempting to say that idiophase prevails, not a trophophase in nature, which is more a culture property of microorganisms.

5.8.3 The Recombinant Products

The invention of recombinant DNA technology expanded the spectrum of possible product fermentation. Microbial cells can be inserted into genes from the higher organisms to allow recipients to synthesize "outside" proteins. A wide variety of microbial cells were used as hosts for the use of *Escherichia coli*, *Saccharomyces cerevisiae*, and *filamentous fungi*, and interhuman serum albumin, epidermal growth factor receptor vIII, growth factor, calf chymosin, and bovine soroatostatilo.

5.9 Scaling Up of Industrial Fermenters

Scale-up is the rise in fermenter level or dimensions, e.g., laboratory scale to pilot scale or a large production scale. Microbial processes are usually conducted for a commercial purpose, primarily to give consumers quality advantages and produce financial returns to investors. A technique produced in laboratories (e.g., fermenters of 0.5–10 L), with a scale range from thousands to millions, has to be transformed into a complete production process (e.g., 20,000–2,000,000 L fermenters). The critical feature to be restrained on the scale-up is the volume of the fermenter. Increasing the surface area by the square function, airflow rate, carbon dioxide, oxygen transfer, agitation rate, and foaming will be affected. If some parameters such as impeller speed endure perpetually at the fermentation process as power output, it surges to a widespread assortment and becomes economically unviable. Thus it is not easy to control the same level of mixing on scale-up as it primes to less satisfactory mixing behavior (Schmidt 2005).

Scale-up can also modify the generation of foam, shear forces, and rate of elimination of carbon dioxide. Scaling up using continuous oxygen transfer rate (OTR) can calculate or evaluate the volumetric mass transfer coefficient, kLa. One set of principles is extensively used to uphold the resemblance of the three different power input parameters per unit volume, geometry, and superficial air velocity. The consequence of the latter is to decline the comparative volumetric airflow rate as scale increases.

If a high degree of originality arises in the process and the commercial product, large industrial processes are ideally accelerated over two levels. The first stage is a pilot plant (pilot scale) with fermenters of 100–10,000 L and equipment upstream. Their goal is to convert the laboratory process into a practical, downgraded version of the production process. In utmost cases, the method is not entirely integrated; e.g., each device's process is operated in a batch manner. The pilot scale selected is a measurement constructed on the measurement, accessibility, and cost of descriptive downstream equipment and the sample sizes required. The second phase of the scale-up is a demonstration plant (demo scale) with fermenters between 10,000 and 100,000 L and downstream equivalents. The risk is to be reduced by further validating the operation, supply chain (from the raw materials to the commercial good application), and consumer demand for significant capital investment in the complete manufacturing plant.

The financial expenditure to increase the production size of a microbial process is typically higher than improving production microbes and laboratory processes. The production plant's annual operating costs are in the same order. Usually, it takes 3–10 years for the transition from laboratory to development. Under those conditions, the financial risk is high. This could be costly and lead to a loss of process efficiency during growth, likely leading to a project failure. So it is sufficient to correct and get it right for the first time when scaling up microbial processes.

5.9.1 Key Considerations for Fermentation Scale-Up

Low fermentation output is always regarded as a priority risk. Fermentation efficiency also affects the enactment of all downstream unit operations and process waste quantity/nature. Fermentation is also an intricate unit procedure (Yang 2011). The output of these parameters is subjected to modification during scale-up. The cost limitations or the equipment's design and size will alter these parameters and directly affect process efficiency and overall plant economy. To minimize fermentation as an increased risk, the realization and attention to scale-dependent fermentation parameters are necessary. For those who follow a consistent approach, large-scale fermentation will be rewarded to meet the output standards and provide good downstream performance. A successful, scalable fermentation, because of the plant's continuous knowledge from years of operational experience, eventually should achieve healthier in the production plant than in the lab. A scaled-down laboratory process diminishes the hazard of performance degradation during the scale-up procedure but does not remove the need for an intermediate scale validation, particularly in the case of a first-of-a-kind procedure. Piloting, usually involving 1–10% of total production, offers specific benefits and reduces more risks:

Process parameter differs as fermenters expand in size and volume, so there are difficulties around the scale-up process. In essence, compromises must be reached in the degree to which other parameters (e.g., speed of pump tip) are constant, for example, if fermentation as a power input is increased widely and economically unviable. Therefore, the mixing level at scale-up cannot be sustained, resulting in a less desirable mixing. Scale-up studies are laboratory studies or even pilot-plant fermenters to generate data for large industrial fermenters' extrapolation and development. Scale-up studies are accessible. In scale studies, several rules are applied as follows:

1. Similarity in geometry and the configuration of fermenters used to scale up.
2. Minimal increase in scaling up of fermentation studies by three or four steps.
3. Each leap in size is increased by magnitude or power and not a few liters. The slight increase in the amount of work does not provide essential data for the operation.

In the case of scale-up, inoculum development and environmental parameters including the availability of nutrients, pH, temperature, dissolved oxygen, dissolved carbon dioxide, and shear conditions are required. Design and development of a pilot plant, including the pilot plant's construction, indicate product economics based on estimated market sizes and competitive pricing and provide guidelines for the permissible production costs.

5.10 Regulation of Industrial Fermentation Via Advanced Instrumentations and Computations

The framework will combine three layers of process control. Each higher level contains more complicated programs and needs to be better understood. The first level of control, used in the chemical industries, is already sequencing, for instance, controlling valves or start or stop pumps, recalibrating instruments, online maintenance, and fail-safe shutdown processes. At least in minutes in utmost operations, high-speed manipulation is not necessary. Sterilization cycles and medium batching are two applications of fermentation processes. The next stage of device control includes temperature control, pH control, foam control, etc., as sensors are interfaced directly with your computer. Separate controller units are not required when this is completed. The program determines the value of the given point, and control algorithms (e.g., PID) are included in the software kit. Enhanced regulator is promising because the control procedures are functions relative than electrical functions mathematically processed. This technique makes a policy of process management more flexible and more detailed. Computer failure will lead to significant problems without manual backup. The alternative solution is to use a computer for the sole control feature. The attached computer records only sensor data and sends signals to modify computer programs and manually change points. This system is called the Set-Point Supervisory Control (SSC). When using the SSC, control modes are restricted to proportional, integral, and derivative, because the electronic controller provides uninterrupted control to the fermenter. However, the process controller is controlled independently in case of machine failure.

Process optimization is the most advanced degree of control. This involves understanding a process, observing what happens, and controlling it to achieve and sustain optimal conditions. First of all, sufficient online sensors are required to track the operation continuously. A few now are usable for dissolved oxygen and specific metabolites (infrared spectroscopy, mass spectroscopy, and near infrared spectroscopy). Second, a mathematical model that effectively designates the dynamic conduct of a process ought to be created. These models' critical role in the batch, fed-batch, and continuous processes for biomass and metabolites has been optimized to enhance yeast development, industrial antibiotic processes, and lactic acid production. Even though a lot is done to regulate a process, few sensors are existing to track the number of metabolites or other parameters in fermentation broth online. To achieve better control, an artificial nerve network is used because of those limitations. Unlike known systems based on experience, neural networks do not require evidence in the method of a collection of rules but acquire from the procedure examples that give them their own rules. This allows nonlinear structures and minimal data to be dealt with. However, it has previously been used in case studies of ethanol production, in the real-time variable estimation, and control of the fermentation and recombinant fermentation of *Escherichia coli*. The system is still at an early stage of growth. There are constricted time constraints for process optimization in industrial systems where vital online and offline data can be

obtainable; the possibility of creating a reasonably accurate model of nerve networks within short time scales becomes very attractive (Glassey 1994).

5.10.1 Monitoring Software

Biomass monitoring and growth rates were developed to calculate the overall energy demand for animal cells in cultivation by online estimating the ATP production rate of oxygen intake and the lactic acid production rate (Dorresteijn et al. 1996). A redox-based software sensor is used to monitor substrate level in *Thiobacillus ferrooxidans* cultures for online estimation of the substrate concentration and biomass. In several cases (San and Stephanopoulos 1984; Iversen et al. 1994; Castrillo et al. 1995), estimated, the introductory rate of growth (μ) based on pH titration was presented. The sensor is constructed on the assumption that the ammonia used for pH checking is converted into biomass with a (relatively) constant proton production per ammonium ion consumed.

5.10.2 Monitoring of Stress Responses in Recombinant Fermentation Processes

Software sensors are not widely used to monitor stress reactions, but increased respiratory activities are a metabolic burden from recombinant protein development. Studies are carried out to track promoters' online regulation associated with stress when fusions with a reporter protein, such as green fluorescent protein (GFP), are used. The monitoring of off-gas from a bioreactor combined with the pattern recognition methods for predicting metabolite and biomass concentration has been carried out with various chemical gas sensors, often called an electronic nose (Bachinger et al. 2001). This method is useful for metabolic burden identification during fermentation processes.

5.10.3 Multi-bioreactor Systems

The need for enormous data and relatively slow rate of ferments are a bottleneck in research into the link between analysis and process efficiency. Many bench-scaling multi-bioreactor systems provide parallel fermentations by several bioreactor manufacturing companies. These systems are ideal for a good experimental design, whereby statistically significant associations are formed between the restrained variable and the progression quality parameters. Also ideal for process optimization is the combination of multi-bioreactor systems and online analyses. Food profiles, induction techniques, and stress builders are essential parameters for process optimism with a multi-bioreactor framework. Multifunctional cultures need a high degree of automation because the job is highly labor-intensive. The automation of fermentation processes requires individual reactor surveillance and control to enable,

for example, automated feed starting or inducing processes. Software sensors may aid the instinctive monitoring of such crops. The requirement for individual reactor control and monitoring results in very widespread data sets for process evaluation (Bachinger et al. 2001).

5.11 Conclusion

Fermenters are used to make a variety of biological and high-value goods. They offer the opportunity to regulate and track the conditions of the fermentation process while also providing additional advantages. Fermenter design affects fermentation rate; many factors such as fermenter size, vessel form, agitation, aeration, baffles, and so on all play a role in output. Various methods for increasing efficiency, as well as various controlling probes, have been developed to improve productivity. To provide a quantitative understanding of heat and mass transfer, appropriate calculation methods have been established. Innovative developments are being implemented in various types of fermenters in order to increase the efficiency rate.

To provide versatile and high-quality bioreactors, reliability and availability on new fermenter designs (bioengineering development) are needed worldwide. The sustainability of the process on a wide scale must consider achieving success in processes and bioproducts. It is important to invest in research and scale-up processes that ensure better performance in the plant's optimum functioning and maintenance. As a consequence of this need, many efficient bioprocesses are established based on the best bioreactor design adapted to a particular production process and one that is economically profitable.

Declaration of Conflicts of Interests The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Strain Improvement of Microbes

6

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Abstract

Strain improvement is an advanced biotechnological strategy where various cellular pathways are modified by recombinant DNA technology to improve the yield of metabolic products that are beneficial to humanity. Strain improvements are directed toward improving product quality and yield by enhancing substrate utilization, regulating enzyme activity, resistance to phage infection, etc. The primary genetic routes to strain improvement include (1) mutagenesis for the creation of genetic variants, (2) screening to select improved strains, (3) identification of improved strains, and (4) mass culture optimization of operational and cellular responses and downstream processing. This chapter details the various strain improvement strategies and the respective computational and biotechnological methods that are used.

Keywords

Strain improvement · Mutation · Gene expression · Microarray · Sequencing · Principal component analysis (PCA)

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_6

Abbreviations

asRNA	Antisense RNA
BWA	Burrows-Wheeler Aligner
CCDS	Consensus Coding Sequence
cDNA	Complementary DNA
CRISPR	Clustered regularly interspaced short palindromic repeats
DDBJ	DNA Data Bank of Japan
EMBL	European Molecular Biology Laboratory
EMS	Ethyl methanesulfonate
EST	Expressed Sequence Tags
ExPASy	Expert Protein Analysis System
GATK	Genome Analysis Toolkit
GI	GenInfo
GO	Gene Ontology
GSS	Genome Survey Sequences
HA	Hydroxylamine
HTGS	High-Throughput Genomic Sequence
indels	Insertions/deletions
MALDI-ToF	Matrix-assisted laser desorption/ionization time-of-flight spectroscopy
MMS	Methyl methanesulfonate
MPSS	Massive parallel signature sequencing
NGS	Next-generation sequencing
NTG	Nitrosoguanidine
PRIDE	PRoteomics IDEntifications
RDT	Recombinant DNA technology
RefSeq	Reference sequences
RISC	*RNA*-induced silencing complex
RNAi	RNA interference
siRNAs	Small interfering RNAs
SNPs	Single-nucleotide polymorphisms
STS	Sequence-Tagged Sites
SVs	Structural variants
TALENs	Transcription activator-like effector nucleases
UV	Ultraviolet
ZFN	Zinc finger nucleases

6.1 Strain Improvement

The practice of fermentation can be traced back to the Neolithic age when ancient people used to ferment food without much knowledge about its science. In modern times, fermentation processes are applied on a commercial scale in different production processes. The chemical changes brought about in different organic substrates by microbial activity convert the substrates into a more valuable product during fermentation. A microbe suitable for a particular fermentation process can have different genetic variants and may be referred to as strains. The naturally occurring, well-characterized strain is taken as a standard reference and is referred to as the wild-type strain. While this wild-type strain might be capable of a suitable substrate-to-product conversion, the process might not be an optimized one. A fermentation process's commercial success requires maximum productivity and quality. To fulfil these criteria, strain improvement is often essential. Thus, strain improvement refers to the inclusion of specific desirable genetic changes via mutations, selection, or genetic recombination (Bhardwaj et al. 2019).

6.1.1 Strain Improvement: Why Is It Important?

The naturally occurring microbes are usually the source of essential molecules required for our survival on Earth. The wild-type strains of such microbes cannot make the molecules of interest in larger quantities. Strain improvement is one way to optimize productivities such that the amount of product yield per vessel per unit time is enhanced. Sometimes, in these strains' metabolic pathways, different metabolites are classified as primary and secondary based on their requirement for the strain's survival. Those metabolites that are not crucial for bacterial growth but are simply by-products that can be of economic importance to humankind are termed secondary metabolites. The targeted manipulation of secondary metabolite production pathway regulators (both positive and negative) can improve the fermentation titers. The development of industrial strains is essential in minimizing the production cost. Some improved strains can function on cheaper substrates, whereas others can maximize productivity by freeing up fermenters for other fermentation products in the pipeline. Strain improvement makes it possible to incorporate specific characteristics for better microbial growth and yields even under adverse conditions. For example, tolerance to various kinds of stress like abiotic stress, toxic products, solvent tolerance, nutritional repression, and anti-metabolite repression are examples of a few of the many desirable characteristics (Fiedurek et al. 2017). To avoid any negative impact of by-products on downstream processing, improved varieties of the wild strain that reduces or eliminates inhibitors' production can be beneficial. Other benefits may include improved genetic stability, bacteriophage resistance, better regulation of enzyme activity, increased permeability, non-toxicity, the removal of undesirable traits, etc.

6.2 Evolution of Strategies in Strain Improvement

Classical strain improvement refers to such strategies as random mutagenesis, directed evolution, and dominant selection (Derkx et al. 2014). These, when combined with some natural ways of gene transfer such as transformation and conjugation, can bring about further variations in their genetic backgrounds.

When natural selection is not possible, random mutagenesis can be used to eliminate specific undesirable characteristics in a shorter time and cost-effective manner. However, a significant downside of this strategy is the accumulation of many unintended and undesirable mutations and desirable ones. Random mutagenesis encompasses several critical factors that have to be taken into consideration. The choice of mutagen (Table 6.1) is an essential factor that has different consequences. Mutagens (Table 6.1) can be classified into physical mutagens, such as UV, gamma, and X-ray radiations, and chemical mutagens such as nitrosoguanidine (NTG), 4-nitroquinoline-1-oxide, mustards (IR170), methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), and hydroxylamine (HA) (Freese et al. 1961; Sikora et al. 2010). These mutagens cause different types of DNA damage and may require different DNA repair pathways. Also, it is vital to have prior knowledge of whether a particular type of mutagen is selectively mutating certain parts of the genome or if it is random. Sometimes the final product is the combined effect of the mutagen and the environmental conditions under which the mutagenesis is carried out. For example, it was reported that the chlorate resistance of *Aspergillus nidulans* depends on the mutability of a set of ten genes. Of these ten, seven mutations resulted from the inability to use nitrate as a nitrogen source. The study showed that chlorate resistance strength depends not only on the mutagen type and dosage but also on the environmental conditions, which in this case were the nitrogen source. Surprisingly, the experiments also showed that despite the high degree of genetic similarity between *Aspergillus nidulans* and *Penicillium chrysogenum*, the identity of the nitrogen source affects the mutation profile in *Aspergillus nidulans*, whereas no such effect was observed in the case of *Penicillium*.

The dose of mutagen must be optimized to obtain the highest number of desirable mutants because a higher dose of mutants might produce desirable secondary mutations while lower doses might be insufficient. Thus, it is important to plot a dosage response plot to depict the mutation induction kinetics (Fig. 6.1). Different

Table 6.1 The damage caused by different mutagens

Mutagen	Damage
UV	Pyrimidine dimers
Far-UV	Hydroxylation of bases
Nitrous acid	Deamination of adenine, cytosine, guanosine
Hydroxylamine	Deamination of cytosine
Nitrosamine	Alkylation
Ethyl methanesulfonate	Alkylation of cytosine and adenine
N-methyl-*N*′-nitro-*N*-nitrosoguanidine	Methylation at high pH

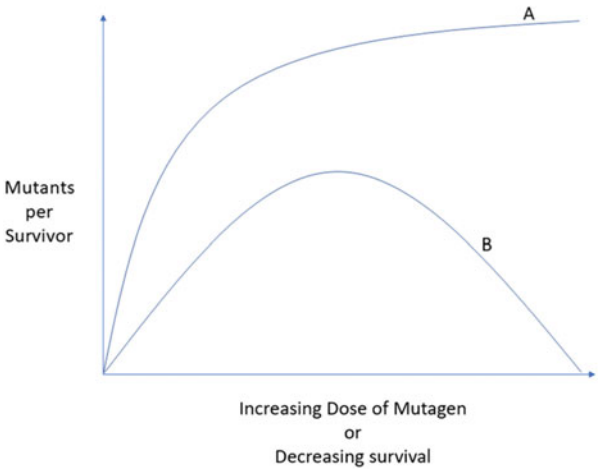

Fig. 6.1 Mutation induction kinetics. Curves A and B depict the kinetics based on the phenotype of the mutation. A can be expected in the case of a mutagen without limiting factors. Curve B can be expected if the mutagen has some limiting factors at higher doses that lead to toxicity

kinds of plots can be expected depending on the phenotype obtained as a result of the mutation induced. It is advisable to choose the dosage that gives the maximum titer improvement in metabolite production. In Fig. 6.1, A and B are the two different types of curves that can be expected based on the phenotype of the mutation scored. For example, if a mutagen has some associated limiting factors, then the optimum titer can be expected at an intermediate dose, higher than which can cause toxicity. B illustrates such a scenario. Without the mutagen's limitations, the maximum growth can be expected at saturation levels with a much higher mutagen dosage, depicted by A.

The enrichment of a strain with specific desirable characteristics requires slow and gradual strain adaptation to a particular set of growth conditions at par with the application parameters to develop the strain by directed evolution in the laboratory. The idea of directed evolution may be rooted in an old experiment known as the Spiegelman's Monster experiment (Mills et al. 1967). In Spiegelman's experiment, RNA was introduced from a bacteriophage Qβ into a solution containing Qβ's RNA replicase, free nucleotides, and salts. After the RNA replication initiation, Spiegelman took out an aliquot of the RNA and transferred it to another tube containing RNA replicase, free nucleotides, and salts. This process was repeated manifold. As shorter RNA chains replicated faster, the selection favored speed. After replication for 74 generations, the original strand of 4500 bases decreased to 218 bases and formed a shorter genome. Thus, the replication of this short RNA sequence was a consequence of the given unnatural conditions.

Recombinant DNA technology refers to the formulation of desirable genetic constructs by manipulating genes (gene knockout, gene knockin, mutation), followed by transferring them into a suitable host. In this manner, it is possible to bring desirable traits together and breed out any negative characteristics (Bhardwaj et al. 2020). For well-characterized organisms, employment of natural recombination systems by sexual or parasexual reproduction was proved to be efficient.

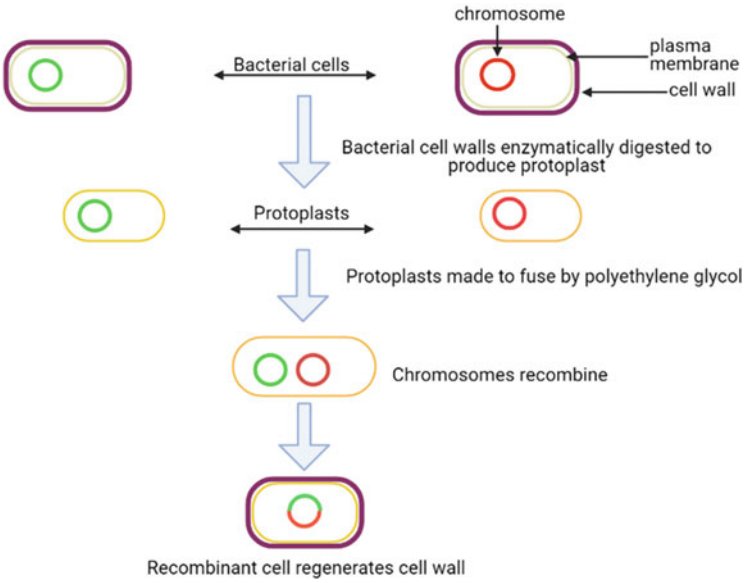

Fig. 6.2 The mechanism of protoplast fusion

However, when there is not enough information about the biochemistry or genetics of production of a metabolite, or when a newly isolated strain is not well characterized, then protoplast fusion is a newer and more flexible approach for creating recombinants (Schaeffer et al. 1976).

Protoplast fusion refers to somatic hybridization, which occurs when two different nuclear genomes that are not genetically close and thus may not produce fertile plants need to be crossed, and sexual crosses are impossible to perform. Strain improvement of low spore-producing strains of certain antibiotics such as *Cephalosporium acremonium* or *P. chrysogenum* has been brought about by protoplast fusion (Adrio and Demain 2006). There have also been cases where a new antibiotic, indolizomycin, was produced by protoplast fusion between nonantibiotic-producing mutants of *Streptomyces griseus* and *Streptomyces tenjimariensis*. The benefits of protoplast fusion include the ability to overcome pre- and postfertilization barriers, transfer of organellar genomes, and male sterility transfer where fertility is unnecessary. A flowchart depicting the basic steps of protoplast fusion is shown in Fig. 6.2.

Natural ways of gene transfer have been utilized in increasing the production of antibiotics like neomycin, kanamycin, penicillin, etc. Transduction is a natural gene transfer system and occurs when a bacteriophage acquires a small portion of the genome or a plasmid from one bacterium and transfers it to another bacteria through viral infection (Harlander 1992). Even though gene transfer efficiency in transduction is high in *E. coli*, the transfer efficiency varies among other bacteria. This

method is used to transfer genes between related species. A drawback to transduction is the lack of characterization of most bacteriophages.

Transformation is another technique whereby if a particular bacterial strain is naturally competent, it can take up DNA from the surrounding medium. Such transformation processes are limited to only a few primarily pathogenic genera, and the gene transfer efficiency varies. If a strain is not naturally competent and recalcitrant to other means of gene transfer, then the use of high-voltage electric pulses of short duration can help induce the formation of transient pores in cell walls and membranes and enable the cells to take up DNA from the surrounding media (Harlander 1992). Bacteria can also be made to gain competency by treating them with a specific concentration of a divalent cation such as calcium, which helps them take up DNA directly. Ultrasound sonoporation is another technique in which cells are forced to induce permeability and take up DNA. It is mainly popular among certain thermophilic strains that are recalcitrant to both chemical and electrotransformation. Protoplast transformation was an improvement that extended the applicability of this technique (Katsumata et al. 1984).

For many gram-positive bacteria strains, the thick peptidoglycan layer often acts as a barrier to DNA uptake. In such cases, enzymatic removal of the cell wall leads to the creation of protoplasts, and these protoplasts can take up DNA in the presence of polyethylene glycol. Transformed protoplasts also, at times, can regenerate the cell walls under optimally stabilized osmotic conditions. This method might be tedious and time-consuming but has been proven to be effective in lactic acid bacteria (Derkx et al. 2014).

Plasmid DNA can sometimes encode important metabolic traits. The natural dissemination of such plasmids occurs when a transmissible plasmid is transferred through conjugation from a donor to a recipient strain. The idea of interspecies conjugation has been utilized in the production of antibiotics like paromomycin, neomycin, and kanamycin between two auxotrophic strains, *Streptomyces rimosus* and *S. kanamyceticus.* Auxotrophy plays a vital role in antibiotic-producing branched pathways, where the antibiotic is the secondary metabolite. In branched pathways leading to a primary and a secondary metabolite, these auxotrophic mutants requiring the primary metabolite sometimes overproduce the secondary metabolite. Alternatively, if the product of interest is an intermediate of the pathway, then end-product feedback inhibition can be ruled out using an auxotroph that requires the end product's utilization for its survival.

In an attempt to rule out the possibility of accumulation of undesirable secondary mutations, the idea of genome-based strain reconstruction can be applied (Ohnishi et al. 2002). In this approach, only specific beneficial mutations are selectively assembled in a single wild-type background. This method was successfully applied in the reconstruction of classically derived L-lysine-producing *Corynebacterium glutamicum* by introducing three-point mutations in the genes of the aspartokinase, pyruvate carboxylase, and homoserine dehydrogenase, which synergistically led to an increase in productivity.

Once a mutation is introduced, its effects need to be monitored at all levels starting from the expression to all the metabolic networks interconnected to the

functionality of the mutant gene. Metabolic engineering refers to the idea of combining analytical methods to quantify fluxes and to control the fluxes with molecular biology techniques to implement suggested genetic modifications (Adrio and Demain 2006). An example depicting this concept's successful utilization was optimizing β-lactam production through heterologous expression of novel genes in well-characterized strains for pathway extension to replace environmentally damaging chemical methods (Thykaer and Nielsen 2003).

To select a particular gene for manipulation, knowledge of all the transcripts (mRNA molecules) that can be produced from that gene is desirable. The massive parallel signature sequencing (MPSS) approach referred to a genome-wide transcript expression analysis and was successfully applied to improve riboflavin production from vegetable oil by the fungus *Ashbya gossypii* (Adrio and Demain 2006; Karos et al. 2004). Fifty-three genes related to vitamin B2 production were identified. Sites with high transcriptional activity for riboflavin biosynthesis were screened out of the genome using the MPSS approach to identify suitable integration loci for the genes.

Combinatorial biosynthesis is another emerging concept in the production of modified drugs. Recombinant DNA techniques were utilized to introduce genes coding for antibiotic synthases into producers of other antibiotics or into nonproducing strains to obtain modified antibiotics (Adrio and Demain 2006). This technique was first brought into successful use when the genes were transferred from a streptomycete strain producing the antibiotic actinorhodin (isochromanequinones class of natural products active against *Staphylococcus aureus*) into strains producing granaticin, dihydrogranaticin (other antibiotics of isochromanequinone class) and medomycin (alpha-6-deoxy-5-oxytetracycline). Combinatorial biosynthesis has also gained popularity for manipulating polyketide genes toward selective enzyme replacement in the biosynthetic pathways to produce new polyketides. Haploid segregants have been used to increase the yield of penicillin. Haploid segregants can be obtained by recombining two second-generation strains derived from a common ancestor possessing two different alleles responsible for high production levels (Parekh et al. 2000).

Other alternatives to natural recombination are DNA shuffling by molecular breeding techniques (Eness et al. 2001). In this case, the concept of homologous replication is mimicked in vitro to recombine DNA fragments and introduce point mutations at a very low controlled rate. This concept of pooling and recombining parts of similar genes from different strains can significantly improve enzymes in a short amount of time. DNA family shuffling or whole-genome shuffling is an extension of this concept.

A notable limitation of restriction enzyme cloning is that sequence modifications can only be made at restriction enzyme cut sites. The lambda red recombineering system, derived from lambda red bacteriophage, allows modification of target DNA independent of cut sites (Murphy 2016). Here modification occurs in vivo, where a linear donor like ssDNA or RNA is used as the substrate, and the set of lambda red enzymes (Gam, Exo, Beta) catalyze its homologous recombination with the target. Gam prevents the degradation of linear double-stranded DNA by the nucleases; lambda exonuclease (Exo) degrades dsDNA from 5′ to 3′ with only single-stranded

DNA left in the recessed regions. Beta binds to these single-stranded regions and facilitates recombination by promoting annealing to the homologous genomic target site.

ZFN (zinc finger nucleases) can induce double-stranded breaks, subject to cellular DNA repair processes. ZFN can be used for targeted mutagenesis and gene replacement at remarkably high frequencies. The zinc fingers are short DNA-binding motifs with an α-helix and two to three β-sheets. An example of a zinc finger motif is a set of Cys_2His_2 bound to zinc. Two cysteines and histidine residues are positioned in a way to bind zinc and stabilize the structure (Carroll 2011).

TALEN (transcription activator-like effector nucleases) are chimeric proteins that are a fusion of TAL effector DNA-binding domain and a DNA cleavage domain (a *Fok*l endonuclease) that can bind to any DNA sequence and generate cuts at specific points in the form of double-stranded breaks. The DNA recognition TALEN is secreted by *Xanthomonas*, which is 34–35 conserved amino acids. This technique is similar to the CRISPR-Cas method and has proved to be much helpful in genome editing.

In the interest of having some efficient tools for nucleic acid targeting, which are also highly programmable, CRISPR-Cas (CRISPR stands for clustered regularly interspaced short palindromic repeats), an endogenous adaptive immune system of prokaryotes, was engineered for gene editing (Donohoue et al. 2018). It is a newly emerging technique in genome editing where directed genetic manipulations can develop a highly optimized strain (Fig. 6.3). Initially, CRISPR-Cas, being a prokaryotic immune system, exhibits acquired immunity toward foreign genetic elements. This system can be manipulated to shut off a target gene with the help of Cas and trigger the DNA repair process. Modern technologies have modified versions of Cas that can activate specific genes instead of cutting.

Even though the overall idea of ZFN, TALENs, and CRISPR-Cas is somehow similar with the nucleases targeting specific parts of the genome to create double-stranded breaks and the repair of those breaks by activation of the DNA repair machinery, CRISPR stands out to be way more efficient (Gutierrez-Guerrero et al. 2018). In comparison to ZFN and TALENs that rely on specific binding modules or domains for their specificity, CRISPR-Cas relies on a single guide RNA for targeting the part of the genome that needs to be edited. On the other hand, it can become tedious and expensive to construct the ZFN and TALEN domains and attach efficiently to a desirable nucleotide stretch. CRISPR-Cas also simplifies the construction of vectors with multiple guide RNAs for multigene targeting. Thus, this method is highly preferable to conventional alternatives. Other methods can target and alter the expression at the posttranscriptional level instead of the DNA level.

asRNA (antisense RNA) technology effectively inhibits gene expression by targeting RNA translation. It either hinders ribosome-binding site interactions with ribosomes or stimulates the target RNA's degradation by ribonucleases by altering its structure. This technology was first applied in *Clostridium acetobutylicum.* A computational algorithm Mfold can be used to design the asRNA based on the association rate between the target mRNA and the asRNA and considering the

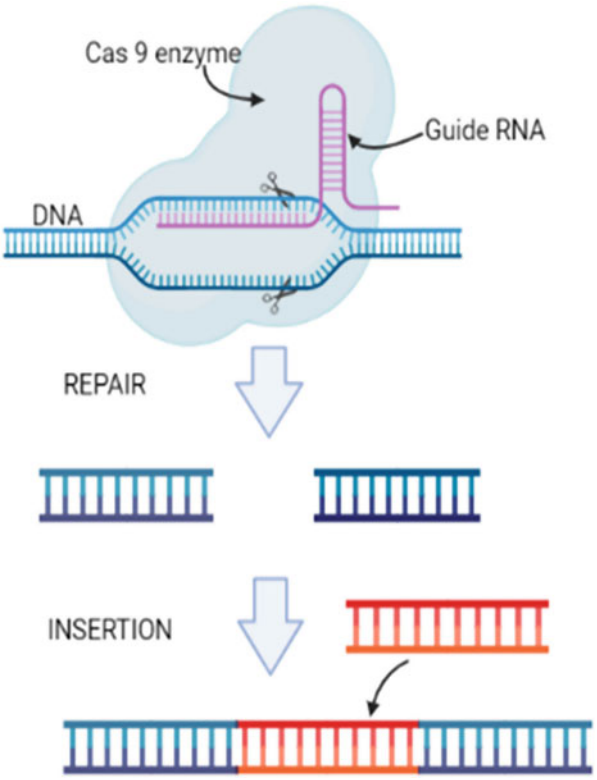

Fig. 6.3 CRISPR-Cas mechanism

secondary structure, kinetics, and thermodynamics of RNA folding (Dong et al. 2011).

RNAi (RNA interference), also known as posttranscriptional gene silencing, is another way to suppress gene expression by activation of ribonucleases. It involves the degradation of dsRNA into small interfering RNAs (siRNAs). These siRNAs are then unwound to form two single-stranded RNAs, the passenger RNA and the guide RNA. The guide RNA is incorporated into the RNA-induced silencing complex (RISC), while the passenger RNA is degraded. This guide RNA of RISC binds to the target mRNA based on complementarity (Agrawal et al. 2003). If the target mRNA and the guide siRNA hold a perfect complementarity, then the target mRNA is degraded by the slicer RNase, a part of the RISC. However, if this complementarity is not perfect, the RISC will also bind to the target mRNA, thus blocking its translation. In either case, the expression of the target mRNA is silenced by RISC.

Plasmids are known to encode many traits important for metabolic engineering and can act as suitable vectors in recombinant DNA technology. An example of this technique's application was the nitrogen-fixing cyanobacterium, *Anabaena* sp. strain PCC7120. Here an integrative expression vector consisting of a noncoding region from *Anabaena* for genomic integration, a strong PpsbA1 promoter from *Anabaena* for expression of the desired gene, and neomycin phosphotransferase gene with its

own promoter for transformants selection was constructed. The cassette was cloned into the suicide vector pBluescript II SK (+) such that cell death occurs if the plasmid does not successfully take up the insert. The entirely constructed resultant plasmid was named pFPN and was transferred into *Anabaena* by electrotransformation (Chaurasia et al. 2008).

Transposons are mobile genetic elements which are DNA sequences that move from one replicon to another with the enzyme transposase's help without host recombination functions. An extensive homology with the site of integration is not required. Transposons have been used for making stable, non-reversing mutants. The method can be used to determine the order of genes and offers easy selection. An example of the widely used conjugative transposon is broad-host-range streptococcal transposons, Tn916 and Tn1545. These have been transferred to a wide range of *Clostridia* for improved consolidated single-step bioprocessing of lignocellulosic biomass into ethanol, methanol, and butanol as biofuel alternatives (Thomas et al. 2014).

Sometimes, in recombinant DNA technology, the process of cloning can be modified by the use of other vectors such as cosmids (containing cos sites of the λ bacteriophage), bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), etc. (Preston 2003). A 34-kb fragment from *Streptomyces rimosus* was cloned using a cosmid and subcloned into pIJ916. The plasmid was then transformed into *Streptomyces lividans* to synthesize oxytetracycline (Binnie et al. 1989). A flowchart showing the general sequence of steps for cloning is shown in Fig. 6.4.

6.3 Functional Genomics: Forward and Reverse Genetics

Functional genomics refers to the functions and interactions of different genes and proteins and how they can work together in a biological system to produce a particular phenotype.

There are two different approaches to functional genomics. One is the forward genetics approach where one encounters a mutant phenotype which turns out to be beneficial in terms of productivity or improved titer, etc., followed by attempts to identify and characterize the gene responsible for the mutant phenotype. The other one is the reverse genetics approach, where a proposed mutation is first induced, followed by which the altered gene expression and overall phenotype are monitored to understand the function of the gene under study. Also, it has to be noted that several associated factors can affect the rates of mutation. CpG methylation, expression levels, replication timing, and GC content can affect the mutational rates. Maximizing expression is the next vital factor to be taken care of once a natural mutant strain has been developed. Some of the factors that might hinder maximal expression are discussed next.

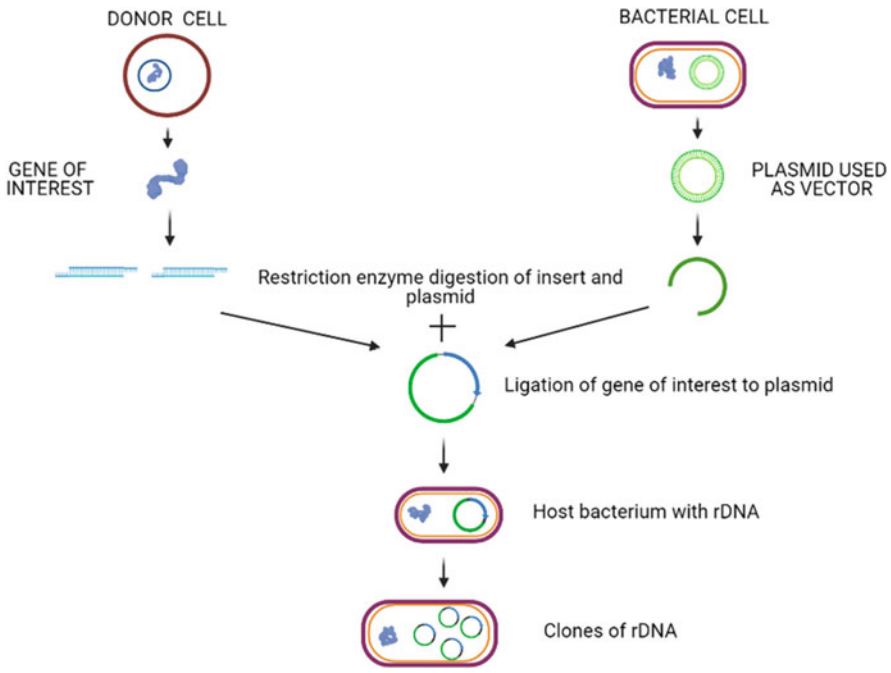

Fig. 6.4 The mechanism of cloning for recombinant DNA technology

6.3.1 Impure Cultures

If the culture used is not a monoculture (i.e., pure culture), it can dilute or suppress the desired mutation. To achieve pure cultures, one must make sure to avoid contaminations. The use of hyphal fragments and multinucleate spores should be avoided. Even with the use of uninucleate spores, all measures should be taken to avoid clumping. In some instances, despite all precautions, the development of mosaicism might be unavoidable. Mosaicism might be observed when a single mutated haploid nucleus can produce a heterokaryotic colony due to a mutation becoming fixed on only one strand of the DNA double helix. Further replication of this DNA produces one mutated and one unmutated daughter cell, giving rise to a mixed colony. Controlling and optimizing the dosage of mutagen can minimize any risks of mosaicism.

6.3.2 Competitive Suppression

In 1952, G.W. Grigg reported that in higher cell concentrations, as a result of removal of a particular energy source by the auxotrophs, fewer prototrophs might be observed, as the growth of prototrophs is suppressed by the presence of sufficiently large numbers of auxotrophs (Grigg 1965). This is also known as the Grigg

effect. This can be avoided if that particular strain can adapt to a different energy source or a reverse mutation from the requirement of a particular component (say an amino acid) to its non-requirement, i.e., prototrophy.

6.3.3 Metabolic Regulation

Metabolic regulation ensures that organisms respond to signals from their environment dynamically. Penicillin biosynthesis in *Penicillium chrysogenum* is under carbon catabolite repression and thus will not occur in the presence of inhibiting concentrations of certain sugars (Magasanik 1961). Penicillin's biosynthesis is regulated by the endo- and exogenous concentration of iron, lysine, valine, and exogenous penicillin concentration (Rowlands 1984). This is an example where a secondary metabolism is controlled by feedback inhibition of the end products of a branched pathway, the primary (amino acids) and the secondary metabolites (penicillin) (Masurekar and Demain 1974).

Also, the products released in the media by the strain of interest broadly impact the medium's viscosity, which will affect the aeration, nutritional requirements, etc. (Lancini and Lorenzetti 1993).

6.3.4 Expression Delay

Expression delay is another factor that needs to be taken into consideration before expecting maximal expression. This involves allowing time for fixation and stabilizing the mutation by DNA repair pathways followed by transcription and translation before the commencement of screening.

6.4 Screening of Mutants

Screening and selection of mutants are crucial once the desirable mutations have been fixed. Random screening refers to inducing a mutation followed by random picking from the survivor population to test their ability to produce the metabolite of interest (Parekh et al. 2000). One drawback of this strategy is that many strains need to be screened to isolate an improved mutant as it is not specific to the mutation-induced. Advanced strategies like fluorescence in situ hybridization have been employed to address industrial strain security.

Another way is through surface culture screens by detecting and measuring a zone of product diffusing out from a colony (Rowlands 1984). Mutagenized cells can be either directly plated on nutrient agar or subcultured onto agar plugs or agar droplets. The plating is followed by zone assays by directly pouring a biological or chemical assay mixture over the colonies and screened for sensitivity to a compound or production of a chromogenic molecule. Even though performing such assays might appear to be simple, they come with a disadvantage. Zone-based screens are

low-resolution screens as it is not possible to detect minor titer differences. As a result, zone-based screens exhibit decreasing exponential relationship between the product title and the zone diameter, such that a significant increase in the titer gives only a relatively small increase in the zone diameter. Use of growth-limiting media, product-degrading enzyme, or shorter incubation periods might help tackle this problem to some extent. Another common problem is that small compact colonies often give exaggeratedly small zones while large diffuse colonies can give exaggeratedly large zones. This problem can be solved by calculating the potency index, where the zone diameter is divided by the colony diameter (Rowlands 1984).

The elution-based assay is comparatively laborious but holds an advantage for better quantification of the product. The underlying idea of elution-based assays is to elute product from the growth medium into a solution which is then assayed. Liquid culture screens are considered high-resolution screens and are advantageous over surface culture screens to be designed to mimic production conditions more precisely. Multilevel screening can be performed in some cases to improve accuracy, with the number of isolates under testing being reduced progressively. Rational screening primarily encompasses the idea of enrichment techniques where the fraction of overproducing mutants is selected out by applying positive selection pressure. This is less tedious than random screening (Lancini and Lorenzetti 1993). Specific distinguishing criteria like morphological differences, resistance to antibiotics, selective detoxification, or test for a biosynthetic precursor can be used for this purpose. When a mutation in a regulatory pathway suppresses a metabolite's production, a subsequent reverse mutation will likely increase productivity.

6.5 Bioinformatics for Strain Improvement

Bioinformatics is the science of data management that can be useful in genetic engineering when molecular biology merges with data-intensive research involving genomics, transcriptomics, and proteomics (Tripathi 2000).

Identifying genes by a comparative analysis of genomes is vital to determine which gene needs to be altered and how to get a desired metabolic phenotype. In parallel to genomics, a study of the mRNA expression levels, and the protein complement of the cells, known as transcriptomics and proteomics, can be carried out by microarray analysis and two-dimensional gel electrophoresis, respectively, and coupled to various mass spectrometry methods (Agrawal et al. 2020a, b; Agrawal and Verma 2020). Analysis of the transcriptome profiles of different strains helps develop an understanding of relative mRNA abundance at different time points and under various conditions. Such analyses give an idea of the possible regulatory circuits and potential genes to be targeted for regulation. High-density DNA microarrays allow monitoring of mRNA in different samples for comparative analysis of wild vs. recombinant strains. Even though an analysis at the proteomic level further helps understand metabolic pathways, the characterization of enzyme activities and monitoring protein-protein interactions is essential (Bhardwaj et al. 2018). A combination of transcriptomics and proteomics is an effective way to map

regulatory mechanisms, as was shown by Lee et al., where they analyzed the transcriptomic and proteomic profiles of *E. coli* W3110 and its L-threonine-overproducing mutant strain (Lee et al. 2003). They reported that the genes involved in glyoxylate shunt, the tricarboxylic acid cycle, and amino acid biosynthesis were significantly upregulated, whereas some ribosomal protein genes were downregulated.

Metabolomics deals with the quantification and profiling of metabolic intermediates and metabolites. Fluxomics analysis is essential for applying in silico techniques to strain improvement (Lee et al. 2005). Flux balance analysis (FBA), based on the basic principle of conservation of mass, plays an essential role in the constraint-based analysis of metabolic flow and simulation of metabolic networks in genome-scale reconstructions. FBA requires only the reaction stoichiometries, the metabolic demands, and some strain-specific parameters (Edwards and Palsson 1998). Without the requirement of enzymatic kinetic data, one can get ideas about the metabolic genotype, phenotype, robustness, and redundancy, and all these together can be very useful in designing an improved strain.

While the construction of a strain based on the genes of interest is possible, sometimes it may be challenging to predict their expression in specific metabolic pathways. For example, often, it is desirable to construct a minimal strain containing the essential genes and deletion of extra genes. However, such deletions may lead to a less robust strain that might not be fully expressed under specific culture conditions. Therefore, it is essential to design in silico metabolic models to evaluate the metabolic characteristics and plan the genetic engineering strategies accordingly (Kumar and Verma 2020, 2021).

6.6 Tools for Metabolic Models

Collating the relevant omics data for metabolic engineering of an improved strain is essential but not enough. The construction of an in silico model in a deterministic and stochastic way is essential to handle the data and formulate feasible algorithms. Computational tools are available to help in strain optimization and metabolism. Some of these tools are discussed next.

6.6.1 E-Cell

E-Cell is a software environment for building integrative models based on gene regulation, metabolism, other biochemical processes, and signaling (Tomita et al. 1999). Simulation of cell behavior is possible in E-Cell by constructing hypothetical cell models by numerically integrating differential equations based on protein functions, protein-protein interactions, protein-DNA interactions, gene expression regulation, and metabolomics.

6.6.2 GEPASI

GEPASI is software that can monitor biochemical reaction networks (Mendes 1993). Different combinations of parameter values define control coefficients for metabolic analysis. The simulation is based on the kinetics of biochemical reactions and provides tools to fit data to models, optimize model function, and perform metabolic control analysis and linear stability analysis.

6.6.3 DBSolve

Keeping in mind the need for dynamic visualization, DBSolve was developed to animate simulation using ordinary differential equations and nonlinear algebraic equations results based on metabolite concentrations such that experiments could be modified according to simulation results (Gizzatkulov et al. 2010).

6.6.4 SCAMP/Jarnac

SCAMP is a general simulation software for metabolic and chemical networks, and Jarnac is the second version of SCAMP that can perform continuous and stochastic simulations (Sauro et al. 2003). The software is compatible with multiple data types and supports dynamic simulation, steady-state analysis, simple stability analysis, and interactive metabolic analysis.

6.7 Analysis of Genetic Sequences

Any approach to strain improvement, random or directed, can benefit from knowing the relevant genetic sequences. Before diving into the analytical part, one must be familiar with some of the basics of sequence data access. A brief idea of the tools required to work with sequences toward strain improvement is discussed next (Raychaudhuri 2006).

6.7.1 Accessing Biological Databases

Sequence information can be accessed from databases like DDBJ (DNA Data Bank of Japan), EMBL Bank (European Molecular Biology Laboratory), and GenBank, among which GenBank is the largest and most rapidly growing database (Baker et al. 2000; Benson et al. 2010; Mashima et al. 2017). Specific DNA and RNA level data can be obtained for Sequence-Tagged Sites (STS) in the Probe database at NCBI (Alm et al. 1996). Genome Survey Sequences (GSS) and Expressed Sequence Tags (EST) may be used for sequences obtained from cDNA (a partial DNA sequence of a

cDNA clone sourced from RNA). High-Throughput Genomic Sequence (HTGS) makes incomplete sequence data readily available to the scientific community.

Apart from gene sequences, the translated coding regions from GenBank sequences and others can be found in UniProt (Consortium 2008b). UniProt is a protein sequence catalog containing three databases, Swiss-Prot (Bairoch and Apweiler 1997), translated EMBL (TrEMBL) (Bairoch and Apweiler 1996), and Protein Information Resource (PIR) (Bairoch and Apweiler 1996).

6.7.2 Optimizing Search Within a Database

For convenient search and information extraction from databases, specific labels have been used for identification. Some of them are accession numbers, unique sequence identification numbers, and GI (GenInfo) numbers which not only help in fetching the desirable sequence but also get reference sequences (RefSeq) and Consensus Coding Sequence (CCDS) as a comparative standard (Pruitt et al. 2007; Pujar et al. 2018).

6.7.3 Comparing Sequences Based on Percentage Similarity

There are computational algorithms designed to compare sequences based on the best match between two (pairwise) or more than two (multiple) alignments, depicting identical, functionally conserved, and non-conserved matches. Later, weight matrices are constructed based on these alignments in a position-specific manner. Any algorithm that aligns sequences has a scoring function proportional to the quality of the alignment that can be optimized.

6.7.4 BLAST (Basic Local Alignment Search Tool)

BLAST is based on linear time pairwise alignment, which is used to search the database for sequences matching with a query sequence. Position-specific iterative BLAST (PSI-BLAST), a refined version of BLAST, combines the speed of BLAST with the sensitivity of weight matrices.

6.7.5 HMM (Hidden Markov Model)

HMM is a probabilistic model used to identify hidden patterns in a sequence of nucleotides in DNA or RNA and amino acids in the protein (Mount 2009). For example, in multiple sequence alignment, HMM is used to move along a particular path from state to state in a Markov chain, matching the sequence given. The following matching symbol is chosen from each state, recording the probability and the probability of going to that state from a previous one.

6.8 Gene Expression Profiling

Once the genetic sequences and other relevant information are fetched, gene expression profiling is the next step (Saghizadeh et al. 2003). By using techniques like oligonucleotide arrays, SAGE, and spotted DNA microarrays, profiling of thousands of genes is possible under varying conditions in the cell at a given time. The array protocols involve spotting cDNA for specific genes at certain positions on a glass slide or synthesis of short oligonucleotide sequence spots on a solid substrate where each spot specifically binds to mRNA expressed from the genes of interest and shows the pattern of genes expressed by a cell at the transcription level by measuring relative mRNA amounts in two or more experimental conditions. Gene expression datasets are analyzed as a collection of genes or conditions associated with a series of expression data in the form of a profile. The profile helps understand how the cell functions under the experimentally chosen conditions because the cell's environment influences the gene expression at a given time.

6.9 Handling Sequencing Data

After Sanger sequencing, next-generation sequencing (NGS) is a revolutionizing technology that has allowed the sequencing of reading lengths of about hundreds of base pairs and processing of a whole-genome sequence for USD 2000 as compared to the earlier sequencing technologies that cost around USD 80 million (Behjati and Tarpey 2013).

Illumina sequencing, which works on cycle reversible termination technology, can generate 1000 gigabases of DNA sequence data in a single run (Behjati and Tarpey 2013). Another popular sequencing technique is pyrosequencing, a proprietary sequencing technology of Ion Torrent (Thermo Fisher Scientific, Waltham, MA, USA) that measures the pH changes when a hydrogen ion is released each time the DNA polymerase incorporates a nucleotide into the DNA strand (Liu et al. 2012). Applied Biosystems' ABI SOLiD™ is another technology that offers a very low error rate by utilizing sequencing by ligation and using a color space approach. In contrast to other sequencing systems (Ondov et al. 2008), SOLiD data are recorded in color space, in which the individual values (colors) within a read provide information about two adjacent bases. The color data is converted to sequence data (decoding) and mapped to a reference genome using conventional alignment tools.

Complete genomics introduced a sequencing platform that generates an average of 45–87-fold coverage per genome by identifying 3.2–4.5 million sequence variants per genome (Drmanac et al. 2010). The fragmented genomic DNA is cloned into vectors such that each single-stranded vector contains hundreds of insert copies and can self-assemble into nanoballs. Sequencing is performed on the principle of combinatorial probe anchor ligation sequencing. A library is constructed with fragmented DNA added to two synthetic DNA sequences or half adaptors at each end of the sheared fragments. The molecules are then circularized by intramolecular

ligation to form a complete adaptor. The second set of half adaptors is added to linearized DNA, and the molecules are recircularized. Thus the library contains around 70 base pairs (bp) from each end of the original genomic DNA fragment, separated by the 2 adaptors. The anchor sequences hybridize to the synthetic adaptors, and a pool of fluorescently labeled probes associate with bases at that position. The probe with the appropriate sequence hybridizes adjacent to the anchor. The ligation of the anchor and the probe stabilizes the interaction. Simultaneously, the unligated probes are washed away, followed by the flow cell's imaging by simultaneous high-speed four-color detection. After a base is read, the entire anchor-probe complex is removed, and the substrate is ready for another cycle of sequencing, using a different combination of probes and anchors to interrogate a different base position.

The appropriate handling of NGS data is crucial. For example, the high titer β-lactam-producing strains currently available in the market were produced due to a strain improvement program that started decades ago (Salo et al. 2015). This involved the next-generation sequencing of the *P. chrysogenum* strains NRRL1951, Wisconsin 54-1255, DS17690, and DS68530 using the Illumina HiSeq platform, followed by quality assessment and metabolite analysis. Genome Analysis Toolkit (GATK) workflow is generally used for NGS data processing (Brouard et al. 2019). Post-DNA library preparation and NGS, quality assessment is vital as there can be various kinds of errors as a function of GC content, homopolymer, and read lengths. The data quality assessment is done with software such as FastQC which also maps the raw reads in FASTQ format to a reference genome. Bowtie is a highly efficient short read aligner that allows fast and memory-efficient alignment of large sets of sequencing reads to a reference sequence, while BWA (Burrows-Wheeler Aligner) is efficient for both short and long reads (Langmead 2010; Li and Durbin 2009). This is followed by realignment, removal of duplicate reads, and recalibration of base quality scores.

The next phase involves variant identification in the categories of insertions/deletions (indels), structural variants (SVs), and single-nucleotide polymorphisms (SNPs). Structural variation can be due to deletion, inversion, mobile-element insertion, novel sequence insertion, tandem duplication, and interspersed duplication, which can be detected at the levels of assembly, paired read analysis, read depth, and split reads. It is crucial to estimate the depth of coverage (read depth) needed to sequence DNA. Higher coverage gives improved statistical power, but it is costly. SAMtools and IGV (Integrative Genomics Viewer) are excellent tools to visualize genomic data (Thorvaldsdóttir et al. 2013). As hundreds of gigabases of DNA sequence can be generated in an NGS experiment per day, large datasets can be stored in different ways. It can be in the form of an external drive that can maintain the data on a local server or be stored in an archive or repository.

6.10 Microarray Analysis

Microarrays are a popular technique for large-scale gene expression analysis where one can map differential expression in terms of upregulation or downregulation of genes (Tarca et al. 2006). Even though microarray advancement is now available for gene expression analysis in the form of next-generation sequencing-based transcriptional profiling, microarray studies are still widely in practice. The basic workflow of microarray is to convert RNA samples into a stable form such as cDNA (complementary DNA) and label it with a fluorescent dye. The labeled cDNA is then hybridized to the microarray surface containing thousands of pre-selected DNA elements.

There are different methods to analyze the microarray data. The normalization of data is essential for making any required corrections before comparing the gene expression. These corrections are required because of the dyes being incorporated into cDNA samples with different efficiencies, differences in DNA quality, washing efficiency, and signal detection. The data normalization ensures the background intensity (blank) subtraction from the signal for each probe set.

6.11 Proteome Analysis

Apart from the comparative analysis of transcriptomes, proteomic analysis of related strains is very important. A protein's identification involves a three-step approach involving direct protein sequencing, gel electrophoresis, and mass spectrometry. A high-throughput chromatography can implement the whole-proteome analysis in combination with mass spectrometry. The GO (Gene Ontology) consortium project deals with interpretation and annotation of microarray experiments that profile RNA transcripts, unification of genes and gene products, and the gene products' organization based on molecular function, biological process, and cellular compartmentalization (Consortium 2008a). A study based on *Saccharomyces cerevisiae* wine yeast's strain improvement showed that differences between the transcriptome and proteome appear to depend on the GO category of the corresponding genes. The study suggests that the enzymes involved in metabolism and their corresponding genes show a strong correlation over time and between strains and suggest strong transcriptional control of these enzymes (Rossouw et al. 2010).

Expert Protein Analysis System (ExPASy) is a freely accessible web server that provides various information required for protein analysis (Gasteiger et al. 2003). Also, an ExPASy Swiss-2D PAGE site includes reference maps from different organisms that help identify individual proteins in 2D gels. Mass spectrometry is a revolutionary technique in proteomics due to its ability to characterize proteins with a high degree of accuracy and precision and detect very subtle changes in protein sequence, such as the addition of a single phosphate. The detector in matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectroscopy records a time-of-flight spectrum based on the mass-to-charge ratio (m/z) of ions which can be used to detect the mass of each peptide fragment (Koenig et al. 2008). A critical

step in mass spectrometry experiments involves identifying proteins by matching the observed mass spectra to the theoretical spectral profiles of peptide fragments obtained from protein databases. Different software tools can do this, like the PRoteomics IDEntifications (PRIDE) database and the PRIDE Inspector software. The PRIDE search results show a list of peptides and their overlap with the given protein sequence $\Delta m/z$ as a measure of quality control, matching peptides, missed tryptic cleavages, etc. Another popular tool in this field is Mascot®, which provides a scoring algorithm and an *E* value similar to BLAST. The software integrates three different search methods based on peptide mass fingerprinting, sequence queries, and MS/MS ion data obtained from peptides (Koenig et al. 2008). The study of posttranslational protein modifications requires the prediction of protein phosphorylation sites. Different machine learning methods and many software packages are available to predict these phosphorylation sites. NetPhos and Scansite use a position-specific scoring matrix that considers various factors such as hydrophobicity of surrounding residues and the kinase specification (Koenig et al. 2008). Such tools have led to remarkable advancements in this field and led to the introduction of phosphoproteome databases.

6.12 Conclusion

With the development of industrial uses, the demand for improved strains is ever increasing. Such improved strains with enhanced metabolic capability can overproduce metabolites for pharmaceutical or food industries and come with improved qualities such as eliminating undesirable traits, better bacteriophage resistance, and stress tolerance. Improved strains can be brought out either by screening wild-type strains from natural sources or genetic engineering of existing strains, as per the industrial requirements. There, however, exist certain risks in this process. For example, sometimes, even in small networks, mutation or knockout of one gene may cause a cascade of unprecedented changes in related genes' expression. Thus, a prior mapping of the entire genetic network around the gene under manipulation is critical. The advancement of analytical techniques like DNA microarray for quantification at the transcriptional level, mapping gene expression at the proteomic level, mapping regulatory elements, and measuring metabolic fluxes have enabled genetic network maps. Such analyses require sophisticated equipment and personnel with significant skill and expertise. Hence, this entire process requires much patience due to the uncertainties in the designed experiments' outcomes. A thorough understanding of the complex networks and hypotheses before undertaking the actual effort to validate the proposed genetic manipulation is required to develop an improved genetically engineered strain.

Acknowledgments This work was supported in part by SERB Core Research Grant, EMR/2016/003705 (SD), and MoE-STARS/STARS-1/643 (SD). AK is supported by a Junior Research Fellowship from CSIR, Government of India.

Conflicts of Interest The authors do not have any conflict of interest to declare.

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Enzyme Kinetics: A Plethora of Information

7

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Abstract

The study of enzyme kinetics has been the fundamental basis of science to understand the reaction mechanism. The most commonly studied model is the Michaelis–Menten equation; however, with the advancement in science and better understanding of the system due to technological advancement, many other kinetic models, such as biphasic, multienzyme, homotropic cooperativity, heterotrophic cooperativity and substrate inhibition models, have been proposed. It has enabled better understanding of the system and also has resulted in the incorporation of multidisciplinary approach. The enzyme kinetics is a collaboration of physics, chemistry, biology and mathematics which has further extended its application in various dimensions along with the understanding of its biological significance. Along with the understanding of the theoretical aspects, the integration of computational approach has further elaborated the understanding of the effect of individual parameters on kinetics and also allowed visualization for better understanding. Thus, considering the above aspects, the present chapter deals with the basics of kinetics, the Michaelis–Menten equation and other kinetics. The multidisciplinary approach as well as the integration of computational approach has been discussed for an overview of the entire topic with respect to current scenario.

Keywords

Enzyme · Kinetics · Models · Multidisciplinary · Computational technique

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_7

Abbreviations

CYP Cytochrome P450
K_{eq} Equilibrium constant
TCA Tricarboxylic acid cycle

7.1 Introduction

The chemical reaction occurs by the transition of the reactant [A] to the product [P] in the presence of the energy barrier, i.e. the free energy of activation and represents the transition state for the conversion of the [A] to [P] (Champe et al. 1994). The transition occurs in the presence of a catalyst (enzyme) that plays a role in lowering the activation energy, facilitating the reaction but does not alter the equilibria of the reaction (Campbell 1993). The enzymes are reaction specific, as a result of which the efficiency of the reaction enhances by numerous folds, e.g. laccase catalyses the oxidation of ABTS (Agrawal and Verma 2019; Agrawal et al. 2020a; Agrawal and Verma 2020). On the other hand, the drug-metabolizing enzymes can act on a broad range of substrates, and this property has been identified as "promiscuity" and can be beneficial for multitude benefits, such as breakdown of drugs in the body to which the body can be exposed under various circumstances in current era. It, however, has to be noted that the specificity of the enzyme is much better than the drug-metabolizing enzyme, where due to broad specificity the number of specific sites becomes less and less (Nagar et al. 2014).

The enzyme kinetics also has its applicability in continuous enzyme reactors (Kamble and Yadav 2017). Further, the increasing use of the bioreactor due to industrialization has resulted in the realization of the role and importance of enzyme kinetics. All the kinetic properties are of utmost importance for the proper functioning of the bioreactor and for understanding the mechanism of action of enzyme. Also, the integration of enzyme kinetics, mass balance and modelling are required to determine and achieve the complete visualization of the operation of the entire bioreactor and for the enhancement of its application potential (Kumar and Verma 2020a, b).

Thus, in the present chapter, the basics of the enzyme kinetics will be discussed.

7.2 Basic of Kinetics

The basic term used in kinetics is rate (k) that represents the speed/velocity of the reaction. It is represented by the unit of the concentration of product formed or per unit time required for the conversion of the reactant. The association and disassociation of the molecules can be designated as k_{on} and k_{off}. As the k_{on} of many reactions

is limited by the diffusion ability of the two molecules to find one another (Creighton 1993), the k_{on} can be used for the determination of the dissociation constant (k_d).

The relation between the formation of the product and k is referred to as the reaction order. The reaction where the product formation is independent from the substrate concentration is referred to as the zero-order reaction. In the case of first-order reaction, linear relationship exists between the concentration of the substrate and the product formation. The unit of the rate constant can be determined as the concentration of the substrate and the rate are already known using the rate expression (Masterson and Hurley 1989). The half-life ($t_{1/2}$) is the time required for the concentration of the substrate to reduce to 50% of its concentration at time zero.

7.3 The Reaction Rates and Order

7.3.1 First-Order Reaction: Irreversible Reaction

The radioactive decay is an example of the irreversible reaction where A is converted to P.

$$[\mathrm{A}] \xrightarrow{k_1} [\mathrm{P}] \tag{7.1}$$

The reaction (arrow) represents that the equilibrium is toward the right and the possibility of Eq. (7.1) being reversible if very small. The rate of reaction/velocity (v) of the reaction can thus be defined as the time required for the conversion of [A] to [P]. As the formation of [P] requires the loss/use of [A], thus, it can be stated as a time-dependent process for the consumption of [A] to form [P], where [A] and [P] also represent the concentration of the substrate and the product, respectively. The conversion of [A] to [P] is unaffected by the concentration as it is an independent event. As the reaction proceeds and [A] converts to [P], its concentration reduces with time (Eq. 7.1), and the rate constant (k_1) has the unit s^{-1} and is the concentration of [A]. As the reaction is an irreversible, unimolecular reaction and relies on the first power of concentration, it has been stated as the first-order reaction, where $[A]_0$ represents the starting concentration at T_0.

$$v = \frac{\delta[\mathrm{P}]}{t} = -\frac{\delta[\mathrm{A}]}{t} = k_1[\mathrm{A}] \tag{7.2}$$

$$\ln = \frac{[\mathrm{A}]}{[\mathrm{A}]_0} = -k_1 t \tag{7.3}$$

$$\frac{[\mathrm{A}]}{[\mathrm{A}]_0} = e - k_i t \tag{7.4}$$

7.3.2 First-Order Reaction: Reversible Reaction

The reversible reaction is represented as Eq. (7.5), and here the equilibrium of the reaction as in the previous case does not lie on one side of the reaction.

$$[\mathrm{A}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\mathrm{P}] \tag{7.5}$$

$$\upsilon = -\frac{\delta[\mathrm{A}]}{\delta t} = k_1[\mathrm{A}] - k^1[\mathrm{P}] \tag{7.6}$$

$$0 = -k_1[\mathrm{A}]_{\mathrm{eq}} + k_{-1}[\mathrm{P}]_{\mathrm{eq}} \tag{7.7}$$

$$K_{\mathrm{eq}} = \frac{k_1}{k_{-1}} = \frac{[\mathrm{P}]_{\mathrm{eq}}}{[\mathrm{A}]_{\mathrm{eq}}} \tag{7.8}$$

The k_i and k_{-1} represent the forward and the reverse reactions, when both the rates of reaction are equal to the consumption of [A] and the overall reaction rate will become zero, i.e. the state of equilibrium $[\mathrm{A}]_{\mathrm{eq}}$ and $[\mathrm{P}]_{\mathrm{eq}}$. It also has to be noted that the state of equilibrium is not altered by the presence of the catalyst as its role is just to increase the rate of reaction where equilibrium can be reached (Eqs. 7.6 and 7.7). The K_{eq} (equilibrium constant) is equal to the rate constant of forward and reverse reaction, and for the reaction to precede the [P] formation, the K_{eq} must be large (Eq. 7.8).

7.3.3 Second-Order Reaction

The second-order reaction is reversible and occurs when two ([A] and [B]) or more reactants react together to form a product [P] (Eq. 7.9). The rate of the reaction depends on the consumption of [A] and [B] to form [P]. The rate constant k_1 has unit $\mathrm{s}^{-1}\ \mathrm{M}^{-1}$, and the reaction has been regarded as second order because rate is proportional to the concentration of the second power.

$$[\mathrm{A}] + [\mathrm{B}] \underset{k_1}{\rightarrow} [\mathrm{P}] \tag{7.9}$$

$$\upsilon = -\frac{\delta[\mathrm{A}]}{\delta t} = -\frac{\delta[\mathrm{B}]}{\delta t} = \frac{\delta[\mathrm{P}]}{\delta t} = k_1[\mathrm{A}][\mathrm{B}] \tag{7.10}$$

Equation (7.10) represents that time (t) is dependent on [A] and [B]. In this case two cases arise, one where the concentration of [B] should be increased in such a way that, when its reaction occurs, only a tiny fraction is consumed and is thus assumed to have negligible change, and the reaction is referred to as pseudo first order (Eq. 7.11), whereas, in the other case, the [A] and [B] are consumed at the same time, i.e. time zero $[\mathrm{A}_0] = [\mathrm{B}_0]$ (Eq. 7.12) (Rogers and Gibon 2009).

$$\upsilon = k_1[\mathrm{A}][\mathrm{B}] = k_1'[\mathrm{A}] \tag{7.11}$$

$$\upsilon = -\frac{\delta[\mathrm{A}]}{\delta t} = k_1[\mathrm{A}]^2 \tag{7.12}$$

7.4 Michaelis–Menten Equation

The Michaelis–Menten equation that plays a very pivotal role in the enzyme kinetics was initially developed by Michaelis and Menten and was later further upgraded by Briggs and Haldane (1925; Michaelis 1913). The equation has two constants, i.e. the Michaelis–Menten constant (K_m) and catalytic constant (k_{cat}). This reaction equation is valid both for simple and complex reactions. Equation (7.13) represents the conversion of [A] to [P] in the presence of enzyme [E], where the first step is substrate binding and the second is the catalytic step.

$$[\mathrm{E}] + [\mathrm{A}] \rightleftharpoons [\mathrm{EA}] \rightarrow [\mathrm{E}] + [\mathrm{P}] \tag{7.13}$$

In Eq. (7.13) the product formation can be stated as the product formation in the term of k_2 (dissociation constant) and is most commonly expressed as k_{cat} (Eq. 7.14).

$$\upsilon = k_{cat}\,[\mathrm{EA}] \tag{7.14}$$

It has been assumed that the K_{cat} and k_2 of the enzyme substrate complex [EA] are slow over the association (k_1) and dissociation complex (k_2). During the reaction of the conversion of [A] to [P], the enzyme substrate complex [EA] reaches a point, where the consumption of [A] and the production of product [P] are equal for a brief time, and this phase where [EA] remains steady (Eqs. 7.15–7.17) for a considerable period of time is referred to as the steady state. After this phase the concentration of the substrate [A] gradually falls and [EA] depletes.

$$\frac{\delta[\mathrm{EA}]}{\delta t} = 0 \tag{7.15}$$

$$k_1[\mathrm{E}][\mathrm{A}] = k_{-1}[\mathrm{EA}] + K_{cat}[\mathrm{EA}] \tag{7.16}$$

$$\frac{k_1[\mathrm{E}][\mathrm{A}]}{k_{-1} + k_{cat}} = [\mathrm{EA}] \tag{7.17}$$

The three-rate constant can be combined as one and can be represented as K_m, i.e. the Michaelis–Menten constant (Eq. 7.18), and Eq. (7.17) can be represented as Eq. (7.19):

$$K_m = k_{-1} + \frac{k_{cat}}{k_1} \tag{7.18}$$

$$\frac{[E][A]}{k_m} = [EA] \tag{7.19}$$

Since, the enzyme catalyses the reaction and also forms the enzyme substrate complex, the concentration of bound and unbound enzyme during the course of the reaction varies; however, the total concentration of enzyme [E_t] remains constant (Eq. 7.20).

$$[E] = [E_t] - [EA] \tag{7.20}$$

As the value of [E] is known, substituting the value in Eq. (7.19) gives Eq. (7.21), which can be further rearranged as Eq. (7.22), and thus substituting the value in Eq. (7.14) gives Eq. (7.23). It has to be noted that the maximum reaction rate (υ_{max}) would be attained when all enzyme is bound to the substrate (Eq. 7.24), and by substituting its value in Eq. (7.14), we get Eq. (7.25). Further, on substituting the value of Eq. (7.25) in Eq. (7.23), the Michaelis–Menten equation is generated as Eq. (7.26) (Rogers and Gibon 2009).

$$\frac{([E_t] - [EA])[A]}{K_m} = [EA] \tag{7.21}$$

$$\frac{[E_t][A]}{K_m} = [EA] \tag{7.22}$$

$$v = \frac{k_{cat}[E_t][A]}{k_m + [A]} \tag{7.23}$$

$$[EA] = [E_t] \tag{7.24}$$

$$\upsilon_{max} = K_{cat}[E_t] \tag{7.25}$$

$$v = \frac{\upsilon_{max}[A]}{K_m + [A]} \tag{7.26}$$

The Michaelis–Menten equation provided the information about the network modeller; however, the K_m, k_{cat} and υ_{max} have to be determined and can be done by measuring the reaction rates (υ). The evaluation of the Michaelis–Menten equation in the form of graphical representation relies on accurate graph fitting, and the plot error associated with non-linear fitting can be avoided using linearization methods, such as Lineweaver–Burk, Eadie–Hofstee and Hanes plots (though they have their own limitations, such as the υ at low concentrations is considerably modified in Lineweaver–Burk and Eadie–Hofstee and to a lesser extent in Hanes plots). Also, the change in enzyme kinetics is greatly apparent due to the presence of inhibitor in the linear plot. Thus, as, in both linear and nonlinear, the plot respective

Table 7.1 The application of the Michaelis–Menten equation in various aspects of science

Sl. no	Application/use of enzyme kinetics	System	References
1.	K_m and V_{max} were determined for compounds using substrate-depletion approach	Recombinant human cytochrome P450 (CYP) enzymes and human liver microsomes	Youdim and Dodia (2010)
2.	ATPase activity assay was according to the Michaelis–Menten Kinetics; then, the parameters were compared with in vitro drug transport study in MDR1–MDCKII Cell monolayers	Human P-glycoprotein (MDR1/ ABCB1) ATPase activity	Shirasaka et al. (2006)
3.	Nernst–Michaelis–Menten model applied to bioanodes formed using sewage sludge	-Bioanodes were formed under constant polarization in 3-electrode set-ups and fermented -Sewage sludge was the inoculum and feeding for the bioanode formation and operation	Rimboud et al. (2015)
4.	K_m and V_{max} determination for xylanase from *Aspergillus oryzae* LC1	Fungal enzyme system, xylanase	Bhardwaj et al. (2019, 2020)
5.	K_m and V_{max} determination for cellulase from *Schizophyllum commune* NAIMCC-F-03379	Fungal enzyme system, cellulase	Kumar et al. (2018)
6.	K_m and V_{max} determination for laccase from *Myrothecium verrucaria* ITCC-8447	Fungal enzyme system, laccase	Agrawal et al. (2019, 2020b)
7.	K_m and V_{max} determination for laccase from *Stropharia* sp. ITCC 8422	Fungal enzyme system, laccase	Agrawal et al. (2020a)
8.	K_m and V_{max} determination for thermostable laccase from *Bacillus* sp. HR03	Bacterial enzyme system, laccase	Nasoohi et al. (2013)
9.	K_m and V_{max} determination of cold-active and salt-tolerant α-amylase from marine bacterium *Zunongwangia profunda*	Bacterial enzyme system, α-amylase	Qin et al. (2014)
10.	K_m and V_{max} determination of lipase 3646 from thermophilic indigenous *Cohnella* sp. A01	Bacterial enzyme system, lipase	Golaki et al. (2015)

errors are associated, the most feasible way to avoid such error would require better understanding of the sample and goal of the experiment in order to effectively detect the source of error (Rogers and Gibon 2009) (Table 7.1).

7.5 Other Kinetic Models

These kinetic models consist of reaction system, where a single enzyme binds to multiple binding sites for either substrates or inhibitors or in the presence of multiple enzyme systems. Thus, the other kinetic models reported in the literature are as follows (Fig. 7.1.):

7.5.1 Biphasic Kinetics

The reaction following the Michaelis–Menten kinetics has saturation velocities as a result; when the substrate concentration becomes greater than K_m, the velocity moves toward zero-order kinetics, as a result of which the increase in substrate concentration does not have a positive impact on the velocity. On the other hand, there are certain reactions where such saturation is not achieved; thus, high concentration of substrate and its reaction with enzyme will give linear increase in the reaction concentration. These reactions are biphasic reactions and are represented as Eq. (7.27):

$$\upsilon = [(V_{max1}[A]) + (CL_{int}[A]^2)]/(K_{m1} + [A]) \tag{7.27}$$

where K_{m1} and V_{max1} are the kinetic parameters for the reactions that are saturable. Now, as in this case, the K_m of the second component is very high and its saturation cannot be achieved, e.g. due to stability thus, the K_m and V_{max} of the reaction cannot be determined as a result it is represented as slope, i.e. CL_{int}. Now, this equation is valid for biphasic reaction and if a reaction follows saturation than CL_{int} will be regarded zero and subsequently Eq. (7.27) reduces to the Michaelis–Menten equation (Korzekwa et al. 1998). Thus, the larger the value of CL_{int}, the larger the deviation from the Michaelis–Menten kinetics will be. The biphasic kinetics are observed in reactions where more than one enzyme having different K_m and V_{max} are present, e.g. heterogenous system such as cytosol. It can also be observed in the reaction consisting of purified enzyme, where the enzyme may exhibit more than one binding site for the substrate (Tracy and Hummel 2004; Wei et al. 2007).

7.5.2 Multienzyme Kinetics

In heterogenous enzyme system where more than one enzyme is involved in biotransformation, for the calculation of the kinetic parameters, the total velocity of the reaction resulting in product velocity will be the sum of the contributions of all the enzymes involve in the reaction. If all the reactions follow the Michaelis–Menten kinetics, the velocity can be represented as Eq. (7.28), where an n number of enzymes are involved in the reaction and every enzyme has its specific K_m and V_{max}.

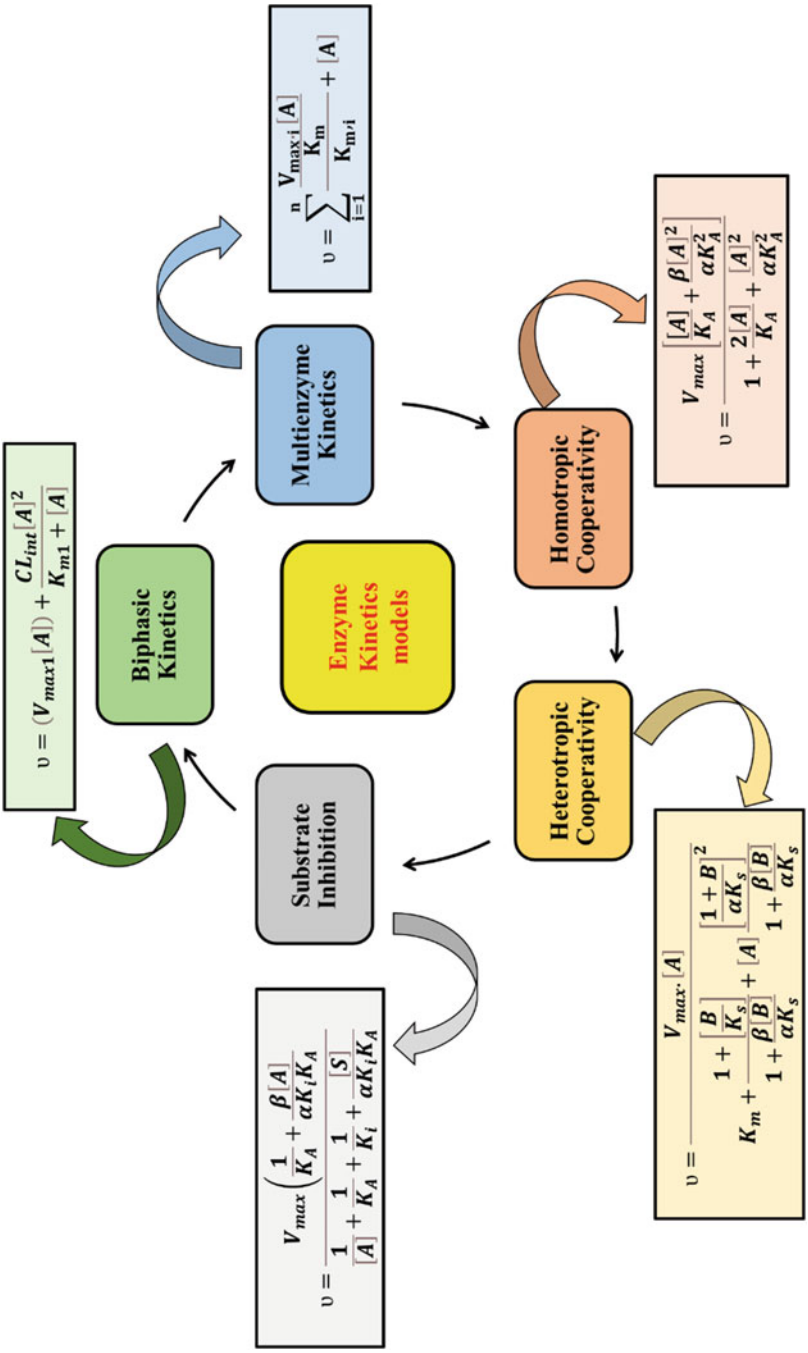

Fig. 7.1 Diagrammatic representation of various kinetic models reported in literature

$$v = \sum_{i=1}^{n} \frac{\frac{V_{\max \cdot i}[\mathrm{A}]}{K_{\mathrm{m}}}}{K_{\mathrm{m},\, i}} + [\mathrm{A}] \tag{7.28}$$

However, it has to be noted that the variation of K_{m} and $V_{\max}$ for various enzymes should be large in order to effectively identify the contribution of each enzyme in the reaction system (Houston et al. 2003). As in the case if the variation is small, the multienzyme kinetics may follow the Michaelis–Menten kinetics despite the contribution of multiple enzymes. Thus, if the enzyme system under study has the presence of multiple enzyme system, the contribution of each enzyme should be determined individually via kinetic parameters.

7.5.3 Homotropic Cooperativity

The allosteric/cooperative interactions are such when the enzyme after binding to the substrate results in the conformation changes in the enzyme, thereby altering the other binding sites of the enzyme. This can be feasible in enzyme with single as well as multiple independent binding sites that favour the Michaelis–Menten kinetics (Segel 1975). The multiple molecules of the same substrate bind to the enzyme simultaneously; it is known as homotropic cooperativity and has been described using the Hill equation (Eq. 7.29):

$$v = V_{\max} \cdot \frac{[S]^n}{A_{50}^n} + [\mathrm{A}]^n \tag{7.29}$$

where A_{50} is the concentration of the substrate at which $V_{\max}$ is achieved and is analogous to the K_{m} (Houston et al. 2003) and n is the degree of cooperativity, and when its value is one, Eq. (7.29) is reduced to the Michaelis–Menten equation. As a result of which, values less and more than one indicated negative and positive cooperativity. The negative cooperativity implies that the binding of the first molecule makes the binding/catalysis difficult for the second molecule. When comparing Eq. (7.3) with the Michaelis–Menten data, the positive cooperativity has a sigmoidal shaped graph when velocity is plotted against substrate concentration, though the deviations are better visible in Eadie–Hofstee transformation.

As the Hill equation (Eq. 7.29) does not give an insight into the interaction of the substrate molecule but only gives information on the homotropic cooperativity. Equation (7.30) can be used to determine K_{m} and $V_{\max}$ values at the individual binding sites (Tracy and Hummel 2004; Tracy 2006):

$$v = \frac{\left[\frac{V_{\max 1} \cdot \mathrm{A}}{K_{\mathrm{m1}}} + \frac{V_{\max 2} \cdot [\mathrm{A}]^2}{K_{\mathrm{m1}} \cdot K_{\mathrm{m2}}}\right]}{1 + \frac{[\mathrm{A}]}{K_{\mathrm{m1}}} + \frac{[\mathrm{A}]^2}{K_{\mathrm{m1}} \cdot K_{\mathrm{m2}}}} \tag{7.30}$$

where K_{m1} and V_{max1} and K_{m2} and V_{max2} represent the kinetic parameters for the first and the second binding site, respectively. The binding of the second substrate molecule and changes in the binding affinity and catalytic rate can be described via the incorporation of α and β, and then Eq. (7.30) can be represented as Eq. (7.31):

$$\upsilon = \frac{V_{max}\left[\frac{[A]}{K_A} + \frac{\beta[A]^2}{\alpha K_A^2}\right]}{1 + \frac{2[A]}{K_A} + \frac{[A]^2}{\alpha K_A^2}} \tag{7.31}$$

where α and β represent the interaction factor for binding and catalysis, respectively, and the equation also represents that the two binding sites of the substrate are equivalent. The variation in α and β in the opposite end represents negative cooperativity, the value of >1 for α represents enhancement in binding affinity of the second substrate and, lastly, the positive value of β represents improvement in the catalytic rate constant.

7.5.4 Heterotrophic Cooperativity

Heterotrophic cooperativity is the binding of the molecules of the substrate and effector to the enzyme, resulting in the alternation in the binding or kinetics, and is represented by Eq. (7.32):

$$\upsilon = \frac{V_{max}.[A]}{K_m + \frac{1+\left[\frac{B}{K_s}\right]}{1+\frac{\beta[B]}{\alpha K_s}} + [A]\frac{\left[\frac{1+B}{\alpha K_s}\right]^2}{1+\frac{\beta[B]}{\alpha K_s}}} \tag{7.32}$$

where B, α and β are the concentration of effector, the change in K_m and the V_{max} binding due to effector binding, respectively (Tracy 2003). The graph generated using Eq. (7.6) is hyperbolic; however, when Eadie–Hofstee approach was used, it did not have any squared terms and did not show any curvature.

7.5.5 Substrate Inhibition

The substrate inhibition occurs when at high substrate concentration the reaction velocity is reduced. This happens when more than one substrate molecule binds to the active site, followed by binding to other active sites, resulting in reduced turnover from the first site, and is represented using Eq. (7.33) (Tracy and Hummel 2004):

$$\upsilon = \frac{V_{max}}{1 + \frac{K_m}{[A]} + \frac{[A]}{K_i}} \tag{7.33}$$

where k_i is the dissociation constant for the binding of substrate to inhibitory site. As this equation does not provide any additional information on the kinetic data, a more complex model has been represented as Eq. (7.34):

$$\upsilon = V_{max}(1/K_A + \beta[A]/\alpha K_i K_A)/1/[A] + 1/K_A + 1/K_i + [S]/\alpha K_i K_A) \quad (7.34)$$

where K_S is equal to K_m, α and β are the factors (K_s and K_i) for the dissociation of the substrates at both the sites and V_{max} is the maximum velocity (Zhang and Wong 2005).

7.6 Enzyme Kinetics: A Multidisciplinary Interest

A reaction involves the binding of enzyme to the substrate and leads to the formation of product over time. The reaction can involve simple or complex conversion with the increase in the number of intermediates or the potential for the reaction to be reversible, resulting in the analysis of enzyme kinetics being complex. The reaction of quantitative enzymology was established by Adrian J. Brown and Victor Henri and later modified by Leonar Michaelis and Maud Menten (Michaelis and Menten 1913; Johnson and Goody 2011; Cornish-Bowden, 2015; Cherayil 2013). It stated that the first transient build-up of the enzyme substrate complex is in equilibrium with the presence of both free enzyme and substrate. Later the reaction enters into the quasi-steady state where the enzyme substrate intermediate accumulates during the initial fast stage however depletes during the slow long-time scale (Briggs and Haldane 1925; Briggs 1925) (Fig. 7.2).

Enzymes are highly regulated system and are a part of very complex network system that is involved with the gaining and diffusion of energy. The detailed understanding of the enzyme kinetics is very crucial for predicting the functional behaviour that arises due to the change in the dynamics of the cell that arises due to the enzymes. The knowledge of the enzyme-substrate interaction, product formation and its role biologically will encourage and develop interest among the students, e.g. the role and function of enzymes, such as α-ketoglutarate, citrate synthase, etc., can only be attained by studying tricarboxylic acid cycle (TCA). Thus, studying the role of enzyme and its biological significance will inculcate interest in students along with better understanding of the enzyme kinetics from biological context. The kinetics is associated with enzymology that is associated with biological and chemical discipline. The biological perspective has been described above, and from a chemical point of view, it has been associated with rate constant, acceleration molecularity, rate, time, nanorheology and viscoelasticity.

The other aspect with which the kinetics associated is the potential of the enzyme to reduce the activation energy barrier of stated in transition state theory (Bearne 2012; Aledo et al. 2003). In the case of the molecular orbital theory, the activation energy barrier of the substrate is reduced by the orbital steering. Further, efforts have been put into the electron (quantum tunnelling) and hydrogen tunnelling to elaborate the rate enhancement, and from a physics perspective, it allows the electrons and

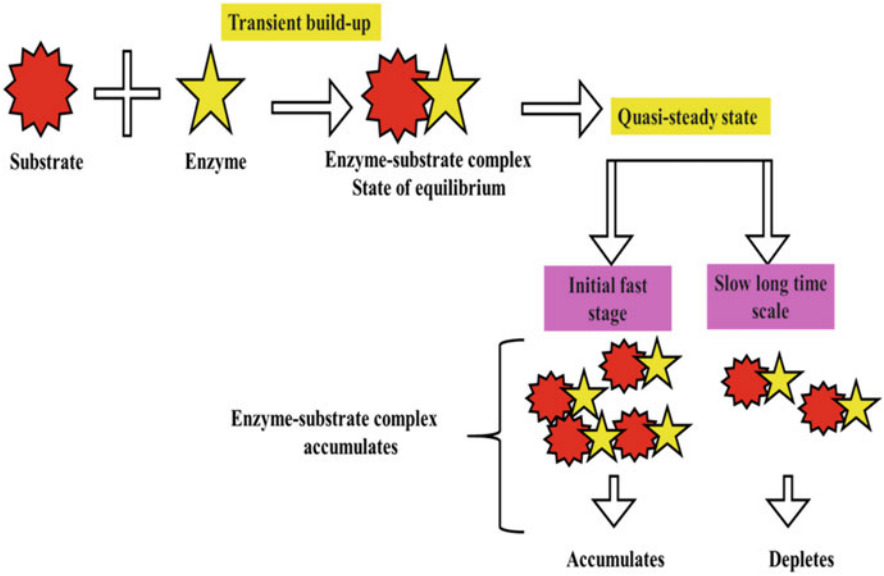

Fig. 7.2 Schematic representation of the reaction of quantitative enzymology

protons to pass through the insurmountable barriers (Devault 1980; Marcus and Sutin 1985; Sutcliffe and Scrutton 2002).

The enzymes are electrical insulators and the probability of the electrons to travel through it is ~3×10^{-9}, whereas, in the case of hydrogen ions, the transfer from the substrate to the product through the tunnelling effect (Klinman and Kohen 2013) (the probability of quantum/electron tunnelling is higher than hydrogen tunnelling as hydrogen is ~1840 times heavier than the electron). Thus, it is very crucial to have the basic understanding of the physics to understand the enzyme substrate interactions in order to completely understand the reaction and its associated mechanism. On the other hand, the reaction is also governed by the covalent and non-covalent interaction; thus, the basic understanding of the biology, physics, chemistry and mathematics is required for the appreciation of enzyme kinetics. The interdisciplinary approach is easy to incorporate but is difficult when implemented at practical level in the classroom. Thus, the structure has to be decided very crucially for effective implementation in the classroom, along with the faculty to be proficient to allow the students to appreciate such an approach that involves the incorporation of enzymology, biochemistry, kinetics, thermodynamics and molecular biology. Thus, the apt balance has to be achieved when adapting interdisciplinary approach so that in-depth knowledge with clear concepts will have positive and long-lasting impact on students (Srinivasan 2020) (Fig. 7.3).

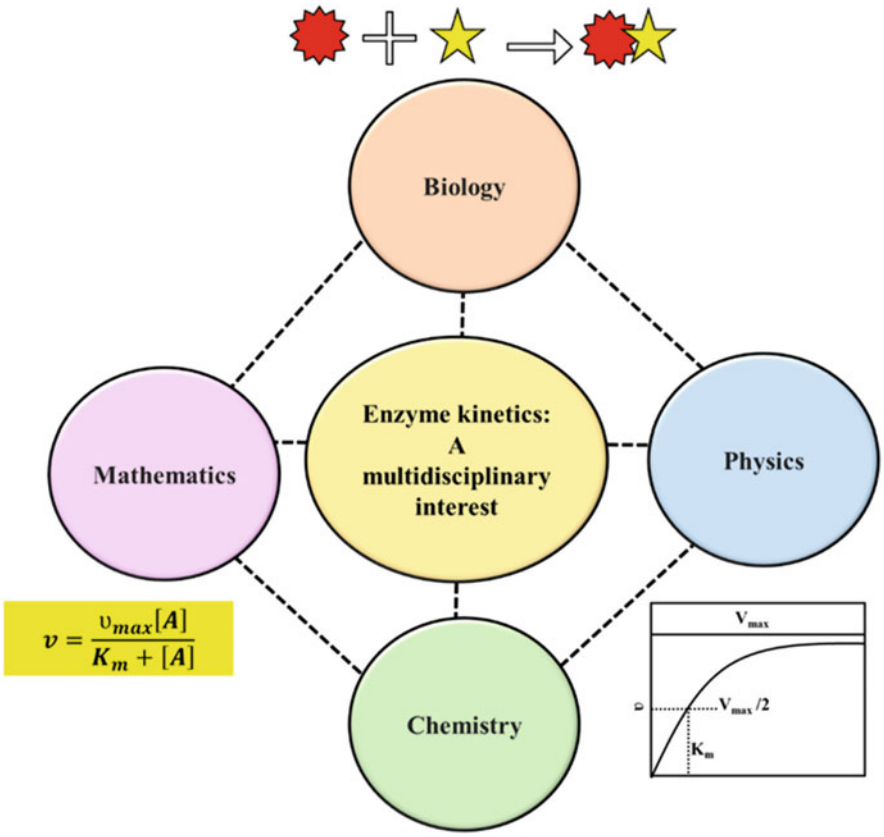

Fig. 7.3 Schematic representation of enzyme kinetics as a multidisciplinary interest

7.7 Enzyme Kinetics: Simulation

The tools, such as KinTek Simulator, Dynafit, KinSim, Gepasi and COPASI (Junker 2010; Johnson 2009; Potratz 2018; Kuzmič 2009; Peng and Jimenez 2019; Mendes 1993; Alves et al. 2006; Hoops et al. 2006), have been used to attain the visual feel associated with enzyme kinetics and to model complete time-course measurements. The role of various parameters can be determined via through the shape of the progress curve. Further, the changes in the parameters and observing real-time change in the build-up of products and depletion of substrates has enabled better understanding of the enzyme kinetics. Also, the simulation of enzyme kinetics enables broader understanding of the topic beyond the classroom with visual feel, which enhances the process of learning. Thus, more detailed study and incorporation of simulation in enzyme kinetics can help in the better and improved learning and understanding of enzyme kinetics (Srinivasan 2020).

7.8 Conclusion

The chapter provides an overview on the various aspects of enzyme kinetics, and other models that have been reported in the literature have been elaborated. The field of enzyme kinetics is immensely diverse and covers and innumerable prospects along with the interdisciplinary approach, and integration of new technologies has paved the way for wide range of studies being reported in literature. The study or the understanding should not only be restricted to the one standard section of enzyme substrate reaction, but its biological significance should also be assessed, thereby opening a wider range of possibilities to be explored during the process of studying enzyme kinetics. Thus, the use of computational techniques, such as simulation, can allow to explore various aspects of kinetics, enabling better and enhanced interpretation of the data.

Conflict of Interest Authors declare no conflict of interest.

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Asparaginase: Production, Harvest, Recovery, and Potential Industrial Application

8

Adityah Ganesh and Surajbhan Sevda

Abstract

Asparaginase is an enzyme that hydrolyzes asparagine to aspartic acid and ammonia. The enzyme finds application in the inhibition of growth and proliferation of tumor cells as the enzyme is broadly used as a chemotherapeutic drug in the treatment of acute lymphocytic leukemia (ALL). Asparaginase also finds application in the food industry in a pretreatment process before oven heating and frying of various foods to reduce the formation and accumulation of carcinogenic acrylamide. The enzyme is produced by several organisms including bacteria, fungi, plants, and animals of which several microbes produce the enzymes at amounts substantial enough for commercial utilization. The majority of the commercial production of the enzyme is carried out using submerged fermentation, while solid-state fermentation accounts for a small margin. The mode of production may be intracellular or extracellular and various among microorganisms. A plethora of separation techniques exist for the purification of the enzymes that are generally classified as low-resolution techniques and high-resolution techniques. Combinations of the two classes of techniques are generally used in commercial extraction. The enzymes extracted from different sources, although similar in function, are structurally different. As a result, asparaginase extracted from different sources varies in the range of temperature and pH values it can tolerate as well as the optimum values for maximum enzyme activity. The enzymes for therapeutic application are primarily extracted from the microbes *Escherichia coli* and *Erwinia chrysanthemum*. The long-term use of the enzyme for therapeutic applications is however limited because of a high rate of

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_8

undesired side effects and hypersensitivity reactions. The enzyme is extensively studied and widely researched upon. Several techniques for enhanced activity, decreased side effects, induced properties, and optimized drug delivery are under development and are expected to be commercially available soon.

Keywords

Asparaginase · Production · Harvest · Recovery · Characterization

Abbreviations

AEP	Asparagine endopeptidase
ALL	Acute lymphocytic leukemia
ATPS	Aqueous two-phase systems
DEAE	Diethylaminoethyl
MALDI	Matrix-assisted laser desorption/ionization
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulfates
SmF	Submerged fermentation
SSF	Solid-state fermentation
WHO	World Health Organization

8.1 Introduction

Asparaginase or monomethoxy polyethylene glycol succinimidyl is an enzyme that hydrolyzes asparagine to ammonia and aspartic acid. The first report of the enzyme was made by Lang in 1904. He reported a hydrolyze enzyme in beef tissue extract which hydrolyzed asparagine to aspartic acid and ammonia (Batool et al. 2016). In 1953, Kidd discovered that serum extracted from guinea pigs regressed Gardner lymph sarcoma xenografts that were implanted subcutaneously in mice forming the basis for the users of the enzyme to treat acute lymphocytic leukemia (Kidd 1953). This was also confirmed by Neuman and McCoy in 1956 who demonstrated that malignant cells were incapable of producing L-asparagine in amounts as high as those produced by healthy cells (Neuman and McCoy 1956). In 1961, Broome showed that asparaginase was responsible for the antilymphoma effect of guinea pig serum (Broome 1961)). Another major achievement that followed was the development and employment of the enzyme as a prime anticancer agent. Asparaginase extracted from. *E.coli* was the first antileukemic enzyme to find clinical application. A modified version of the enzyme was developed in the 1970s in which L-asparaginase was covalently linked to polyethylene glycol (PEG), and the modified enzyme was approved for clinical trials in the 1980s (Bonthron 1990). The PEG association enhances the retention time of the drug in the body. Several side

effects including decreased serum insulin in the pancreas, diabetic ketosis, urticaria, bronchospasm anaphylaxis, and immunosuppression have been reported on the use of the enzyme for therapeutic applications. The use of asparaginase in the treatment of acute lymphocytic leukemia was approved in 1978 and was incorporated in the 18th World Health Organization (WHO)'s list of essential medicines in 2013. The potential use of asparaginase in the control of acrylamide levels in heated foods was recognized, and monographs on L-asparaginase were included in the WHO food additives series in 2008 and 2009, respectively (WHO 2009).

8.2 Sources of Microbial Asparaginase

Asparaginase is distributed in a range of living organisms including animals, plants, and microbes. The microbial sources of the enzyme are usually preferred for their commercial production because of the availability of convenient extraction and purification methods, as well as their ease of handling and maintenance. The major sources of the enzyme are as given in Table 8.1.

8.2.1 Bacterial Sources

Most asparaginase-producing bacteria belong to the family *Enterobacteriaceae*. Intracellular secretion of asparaginase is more common among bacteria, and only a few species are known to exhibit extracellular secretion of the enzyme. This extracellularly secreted enzyme is preferred for commercial production as abundant quantities of the enzyme can be produced in the culture under normal conditions making their downstream purification easy and economical. Similarly, unlike gram-negative bacteria, the gram-positive bacteria secrete enzymes extracellularly into the medium. This is due to the lack of periplasmic space in the case of the gram-positive bacteria. The secretion of enzymes into the surrounding medium thus provides them with an advantage over gram-negative bacteria in bioprocessing and is usually preferred over gram-negative bacteria for commercial production. *E. coli* and *E. chrysanthemi* are currently the primary sources of the enzyme for clinical applications (Batool et al. 2016). *E. coli* produces two types of enzymes, namely, asparaginase I (ECI), which is of cytoplasmic origin, and asparaginase II (ECII) which can be isolated from the periplasm. These two are similar in terms of mechanism of action but differ in terms of their pharmacokinetic interactions and immune system sensitization profile. ECII is used for clinical applications due to its higher affinity (K_m = 10–15 μM) to the substrate (asparagine) compared with ECI (K_m = 3.5 mM). The enzyme may also cause unintended adverse effects such as nausea, allergic reactions, pancreatitis, and coagulation abnormalities due to the glutaminase activity associated with it (Vimal and Kumar 2017).

Table 8.1 Major sources of asparaginase (Source: Modified from Chand et al. 2020)

Type of organism	Major producers
Bacteria	*Escherichia coli* *Erwinia chrysanthemi* *Erwinia carotovora* *Corynebacterium glutamicum* *Pseudomonas aeruginosa* *Pseudomonas fluorescens* *Pseudomonas stutzeri* *Helicobacter pylori* *Mycobacterium tuberculosis* *Staphylococcus* sp. *Vibrio succinogenes* *Pyrococcus furiosus* *Bacillus aryabhattai* *Bacillus licheniformis* *Nocardiopsis alba* *Acinetobacter glutaminasificans* *Acinetobacter calcoaceticus* *Serratia marcescens* *Pectobacterium carotovorum* *Photobacterium* sp.
Fungi	*Aspergillus oryzae* *Aspergillus niger* *Aspergillus aculeatus* *Penicillium brevicompactum* *Penicillium digitatum* *Penicillium* sp. *Aspergillus terreus* *Cladosporium* sp.
Actinomycetes	*Streptomyces ginsengisoli* *Streptomyces gulbargensis* *Streptomyces noursei* *Streptomyces parvulus* *Streptomyces thermoluteus* *Streptomyces* sp. *TA22* *Streptomyces albidoflavus* *Streptomyces aurantiacus* *Streptomyces griseus* *Streptomyces karnatakensis* *Streptomyces venezuelae* *Thermoactinomyces vulgaris* *Streptomyces fradiae* NEAE-82

8.2.2 Fungal Sources

Fungi are the second-largest producer of commercial asparaginase. The enzyme is produced extracellularly and is easy to purify, thus making its commercial production economical. The industrial production of asparaginase from fungal sources is expected to overtake that from bacterial sources due to their superior economic viability and high yield of production. Further, the enzyme extracted from fungal

sources is expected to be more suited for application in the health-care industry compared to that extracted from bacterial sources due to the former being more evolutionarily closer to humans. The enzyme isolated from fungi is expected to have lower cross-reactivity and immunogenicity. *A. oryzae*- and *A. niger*-based asparaginase are already being used as food processing agents in the food industry (Hendriksen et al. 2009).

8.2.3 Yeast Sources

Various genera of yeast have been reported to produce asparaginase including *Saccharomyces*, *Rhodotorula*, *Trichoderma*, *and Rhodosporidium*. Yeast-based L-asparaginase has also been reported to exhibit antitumor activity (Chand et al. 2020).

8.2.4 Actinomyces Sources

Asparaginase has been reported to be produced from actinomyces sources like *S. karnatakensis and S. venezuelae*. Asparaginase activity has also been reported in cell-free extracts of *Thermoactinomyces vulgaris* (Mostafa 1979).

8.2.5 Algal Sources

Reported algal sources of asparaginase include *Chlamydomonas species* and *Vaucheria uncinate* (Batool et al. 2016)

8.2.6 Plant Sources

Some plants including *Arabidopsis thaliana*, *Pisum sativum*, and *Phaseolus vulgaris* have been identified to produce asparaginase (Singh et al. 2017).

8.3 Asparaginase Production

The commercial production of asparaginase occurs primarily through microbial fermentation and this is carried out using two different methods. These are submerged fermentation (SmF) and solid-state fermentation (SSF).

8.3.1 Submerged Fermentation

Submerged fermentation is a proven and widely used process for the large-scale production of various enzymes. Recombinant strains of the microbes *E. coli* and

Erwinia carotovora are the most used microbes in submerged fermentation. Submerged fermentation technology optimized for the growth of microbes like *B. licheniformis* and the fungi *Aspergillus aculeatus* is currently being developed due to the significant asparaginase activity (194.85 U mg^{-1} and 207 U mg^{-1}, respectively) they exhibit, making them suitable candidates for use in submerged fermentation. It is, however, an expensive process due to the need of specialized media like Luria-Bertani (LB) medium, Tryptone glucose yeast extract broth, and modified Czapek-Dox medium as well as the need for advanced equipment (Sudhir et al. 2016).

8.3.2 Solid-State Fermentation

Solid-state fermentation is a cost-effective alternative to the expensive submerged fermentation process. Over the past decade, solid-state fermentation with low-cost agro-industrial residues has emerged as a viable alternative to submerged fermentation (SmF). The process is economical primarily because of the availability of cheap and inexpensive materials for media including various agricultural waste materials such as Bengal gram husk, red gram husk, coconut, and groundnut cakes. The primary limitation of SSF is the lower thermal conductivity of the process as well as the inadequate amounts of free-flowing water in the reactor. These limitations hinder the scale-up potential of the solid-state fermentation process. SSF also has several advantages including higher productivity and lower catabolite repression in the flask level. Because of these, SSF is being studied and modified extensively to optimize it for the commercial scale. The highest reported asparaginase production was achieved using solid-state fermentation using *Bacillus circulans* (Hymavathi et al. 2009). Red gram husk was used for this process and gave an enzyme yield of 2322 U gds^{-1}. Solid-state fermentation can be carried out using a variety of bioreactors. Some of these include koji fermentation, zymotic bioreactor, rotating drum bioreactor, and tray bioreactor. Tray reactor is particularly appealing because of the static nature of the bioreactor and the absence of energy-demanding mechanical parts. Tray reactors have also been extensively studied and optimized for asparaginase production. Multiple rotating drum bioreactor is another recently developed technique and consists of multiple rotating drum bioreactors that are arranged horizontally, with each reactor provided separate inlet feeds. A SSF process using multiple reactors with immobilized cells with polyurethane foam as inert support is a solid-state fermentation system that is being developed for high flexibility and high efficiency (Fig. 8.1). Another major challenge in the use of solid-state fermentation is the difficulty in evaluating microbial growth and estimating biomass production. This is because, unlike in submerged fermentation, where biomass can be directly separated from the medium by techniques like centrifugation or filtration, the biomass (especially fungal biomass) adheres tightly to the solid substrate mixture, often penetrating deep into it, thus making separation difficult and biomass estimation challenging (Mishra 2006).

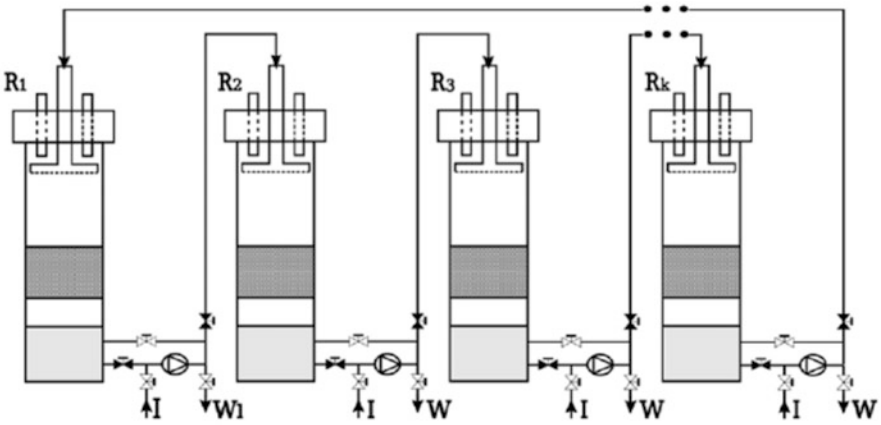

Fig. 8.1 SSF process using multiple reactors with immobilized cells with polyurethane foam as inert support. (Source: Vieira et al. 2020)

8.4 Factors Affecting the Production of Asparaginase

The yield and productivity of asparaginase vary significantly with the strain, fermentation media used, as well as the production process adopted. No specified general conditions or medium has been developed for the optimized production of asparaginase from different microorganisms. The conditions vary for different organisms. Factors affecting the productivity of the enzyme include temperature, pH of culture medium, fermentation time, oxygen transfer rate, and nutritional parameters like carbon and nitrogen sources.

8.4.1 Effect of Temperature

Temperature has a considerable effect on the yield of asparaginase. It has been observed that most of the microbes with a significant asparaginase activity operate in the temperature range between 25 and 37 °C. Studies have reported a notable decrease in production by up to 32.19% with varying the temperature by just 10 °C from 37 °C to 47 °C. However, the optimal temperature for all microbes with asparaginase activity does not fall within this range. The thermotolerant microbe *Streptomyces gulbargensis* exhibits maximum enzyme production at a temperature of 40 °C, while the fungi *Fusarium equiseti* belonging to the ascomycetes exhibited maximum activity at temperatures between 40 °C and 45 °C and are potential candidates to produce heat-resistant asparaginase enzyme which can find applications in the food industry (Amena et al. 2010).

8.4.2 Effect of pH

In the case of most bacteria, maximum asparaginase production was observed at a neutral pH of 7. Fungi had a similar optimal pH range between 6.2 and 7.5. Actinomycetes showed optimal activity between 7.5 and 8.5. The pH of the culture medium affects both the production mechanism of the enzyme and the transport of the various components across the cell membrane. Hence, the control of the pH of the production medium is very important during commercial production. During the commercial production of the enzyme, the medium containing suitable carbon sources (like fructose, mannitol, or glucose) often becomes acidic due to the production of acids during the fermentation processes. This drop in pH affects the yield of asparaginase. Thus, the pH of the medium needs to be continuously monitored and controlled (Alrumman et al. 2019).

8.4.3 Effect of Carbon Source

A variety of carbon and nitrogen sources can be used in the growth media for the microbial production of asparaginase. The vast microbial population with asparaginase activity can ferment a variety of carbon sources like glucose and mannitol. For instance, *Enterobacter cloacae* can use asparagine as the sole carbon and nitrogen source. It can also successfully utilize combinations of maltose, sucrose, fructose, and galactose. The enzyme production is significantly affected by the organism used and its ability to utilize the carbon source. *B. velezensis* showed a 9.3% increase in the production of asparaginase in the presence of glucose in the media. This was observed to be the highest increase with the addition of a carbon source, while the most significant decrease in the enzyme production was observed with the addition of starch and dextran into the media, which reduced the production of the enzyme by 29.26% and 34.69%, respectively. Most other carbon sources did not have a significant impact on cell growth or enzyme production. The plant pathogen *Pectobacterium carotovorum* expressed a very high production when provided with an optimized production medium containing glucose, yeast extract, peptone, and asparagine, while the gram-negative *P. aeruginosa* expressed optimized high productivity in media containing the much less specific 22 g L^{-1} soybean meal (Mukherjee et al. 2000).

8.4.4 Effect of Nitrogen Source

Similarly, the nitrogen sources are also versatile and include asparagine, urea, yeast extract, ammonium sulfate, ammonium chloride, casein, sodium nitrate, and potassium nitrate. When the mesophilic soil bacteria *B. licheniformis* were grown in media containing different nitrogen sources, it was found that ammonium sulfate was the optimum source with a 35.56% higher production than average, while nitrate salts of sodium and potassium were found to reduce the production of the enzyme by

more than 71% of the maximum observed value. At the same time, the addition of urea, yeast extract, and casein was found to have no significant change in the enzyme production of the organism at all. Thus, the choice of the carbon and nitrogen source in the media for the optimization of the production of the enzyme depends on the organism being used. In general, the most common carbon sources used are glucose, starch, and maltose. Asparagine and L-glutamine are widely used as inducers to produce the enzyme.

8.4.5 Effect of Incubation Period

Most bacteria show optimum incubation periods between 24 and 72 h. The highest reported incubation time is 96 h and has been reported in *Pseudomonas aeruginosa* strain 50,071, while the lowest incubation time reported is 10 h and has been reported in the strain *Staphylococcus aureus* NCTC413. The production curve for *Bacillus licheniformis* showed a gradual increase in activity (up to 30.84%) during the first 36 h followed by a phase of exponential increase by 72 h before the drastic fall in the enzyme activity afterward. Actinomycetes have been generally observed to follow a much longer incubation period. The maximum enzyme production of 9.79 U mg^{-1} was observed in *Streptomyces brollosea* NEAE-115 grown in asparagine dextrose starch media after 7 days of culturing. The incubation period varies with the immobilization of the cells. During a study of the kinetics of asparaginase production using *B. velezensis*, it was observed that freely suspended cells had a gradual growth for 48 h, while the immobilized bacterial cells expanded progressively for 72 h. This translates to a 61.04% higher performance in terms of growth. It was also detected that extension of the incubation time beyond the ideal period resulted in a reduction in enzyme production.

8.4.6 Effect of Dissolved Oxygen

Another major factor that influences the production of the enzyme is the amount of dissolved oxygen available. High dissolved oxygen content is often preferred. Aqueous growth medium systems are estimated to be 14% more oxygen-demanding than conventional aerobic processes. Oxygen vectors can be used to increase the dissolved oxygen available to the microbe. The oxygen vector droplets can form an interface between the cell and air bubbles thus enhancing oxygen transfer. Several hydrocarbons like paraffin, n-dodecane, and silicone oil can be used as oxygen vectors. *E. coli* grown in a media containing dissolved oxygen levels above 80% showed a 21% increase in the asparaginase activity when 6% n-dodecane was added to the media. Similarly, a 21.2% increase was observed in the production of the enzyme by *B. brevis* when 6% liquid paraffin was added to it. It was observed that the concentration of dissolved oxygen initially dropped constantly to 6.4% within 6 h. The dissolved oxygen levels were maintained well above 27% when 6% liquid paraffin was added. This was also accompanied by a general increase in the cell mass

of the bacteria. The concentration of the oxygen vector added had a considerable impact on asparaginase production. It was observed that the enzyme activity initially increased with an increase in the oxygen vector concentration until a peak concentration was reached after which the activity starts to decrease gradually. This optimum concentration for both paraffin and dodecane was observed to be 6% (Narta et al. 2011). A similar pattern was also observed in the cell mass production which initially increased, peaked, and then started reducing with an increase in the concentration of the oxygen vector. The negative impact associated with the use of high concentrations of the oxygen vectors may be due to the significant increase in the viscosity of the media associated with it. Increased viscosity harms the oxygen transfer rate of the media (Li and Chen 1994).

8.5 Purification and Characterization of Asparaginase

Asparaginase enzyme has various therapeutic applications and requires a high level of purity. This demands a variety of high-efficiency downstream processing steps which are often complicated and expensive. The purification of the enzyme contributes to up to 80% of the total cost of production of the enzyme. Further, as the properties and yield of the enzyme produced from different sources are different, one standard protocol for the separation and purification of the enzyme cannot be adopted. Generally, it is easier to purify the enzyme of extracellular origin as compared to that of intracellular origin. Similarly, the amount of purity required is also a decisive factor. The therapeutic-grade enzyme demands around 99% purity and requires extensive purification employing multiple highly effective techniques, while the food-grade enzyme requires a lesser amount of purity. This has thus been an area of wide-scale study and research. Various innovative cost-efficient steps for the purification of the enzyme have been developed which serves as an alternative to traditional methods of separation. The efficiency of the new purification methods is monitored continuously by determining the specific activity, yield or recovery, and purification fold after each step. Specific activity defines the total enzyme activity per unit of protein, and its measurement helps study the effectiveness of each step of purification. The yield determines the amount of enzyme activity retained compared to that of the crude enzyme. This too determines the effectiveness of each step and also provides information about any enzyme denaturation or inhibition that might have occurred in each step (Fig. 8.2).

The purification fold defines the quantitative increase in the enzymatic purity at each step and is determined by dividing the specific activity of the enzyme after each step by the specific activity of the initial crude extract (Tundisi et al. 2017). The various methods used for protein purification are discussed below. Often, combinations of different methods are used in specific sequences for the effective purification of the enzyme. The methods and combinations used depend on the desired properties of the enzyme, cost of application, and scale of production. Commonly used combinations of purification methods for the asparaginase enzyme include low-resolution methods like centrifugation, membrane separation, and

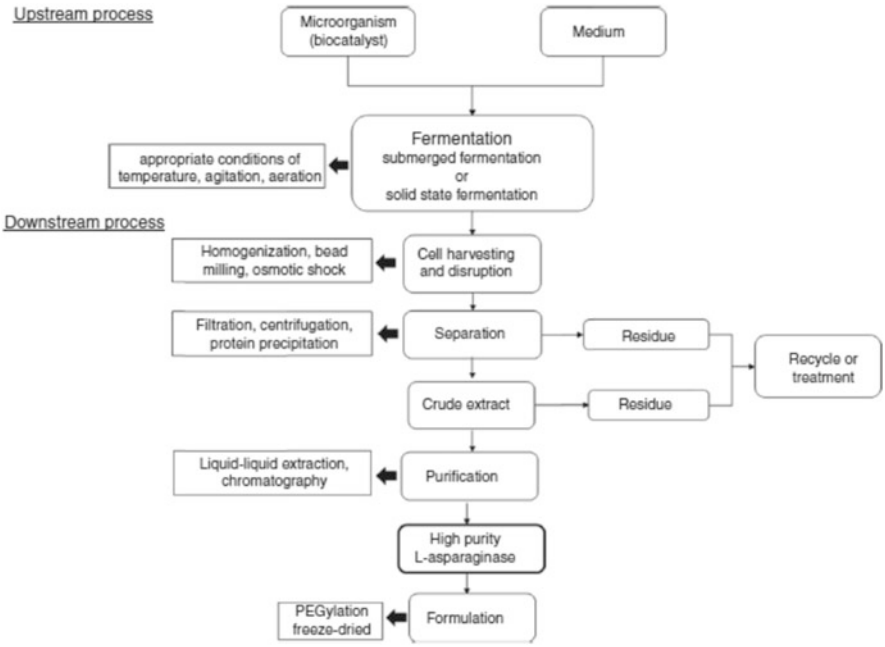

Fig. 8.2 Schematic representation general process of asparaginase production. (Source: Cachumba et al. 2016)

fractional precipitation, aqueous biphasic systems followed by high-resolution techniques like ion exchange, and affinity and gel filtration chromatography. The low-resolution techniques are often called non-chromatographic techniques while the high-resolution techniques are named chromatographic techniques (Badoei-Dalfard 2015).

8.5.1 Low-Resolution Techniques

8.5.1.1 Cell Disruption

Various methods can be used for the disruption of the cell to extract intracellular asparaginase. These include mechanical methods which include sonication, freezing, and grinding; physical methods like osmotic shock; and the addition of chemicals like tween80, SDS, toluene, and ethyl acetate. In a study comparing the abovementioned methods for three potential asparaginase producers, namely, *E. coli*, *Aspergillus terreus*, and *Leucosporidium muscorum*, it was concluded that sonication was the better cell disruption method among the lot for *E. coli* and *A. terreus* (Costa-Silva et al. 2020).

8.5.1.2 Centrifugation

This is a preferred method in the commercial production of the enzyme as it is very efficient and offers only a marginal loss in enzyme activity. The centrifugal extraction of asparaginase was reported which was used to separate the extracellular enzyme from *Streptomyces fradiae* (strain NEAE-82) by centrifuging at 11,000 × *g* for 30 min (El-Naggar et al. 2014). A similar technique was also reported by Hanif, who centrifuged the production media at 10,000 rpm for 15 min at 4 °C for separation using a microcentrifuge.

8.5.1.3 Precipitation

The precipitation of enzymes is carried out by the addition of precipitation agents. The most used precipitation agent is ammonium sulfate, for the purification of asparaginase. Various concentrations of ammonium sulfate have been reported to be effective. Asparaginase was purified from *Pseudomonas stutzeri* using 35–55% saturated ammonium sulfate fraction; further 45% ammonium sulfate was used for the purification of asparaginase from *Nocardiopsis alba* (Manna et al. 1995). Organic solvents are another set of chemicals commonly used for the precipitation of proteins. Among the organic solvents, the use of methanol and ethanol is well established. Kumar et al. reported a very high activity in asparaginase extracted using methanol as compared to other precipitation agents (Kumar et al. 2014). Major challenges in the use of precipitation agents for enzyme purification are their limited selectivity and the possibility for irreversible inactivation of asparaginase during the process. Viable alternative methods and method integrations like ATPS (aqueous two-phase systems) and membrane process integration methods are being developed as a solution to these drawbacks. ATPS are versatile and can be designed with two different chemically dissimilar polymers or with a specific salt and a polymer. PEG-phosphate is the ATPS most used in protein separation. Up to 90% recovery of periplasmic asparaginase was achieved using a combination of ammonium sulfate precipitation and a polymer salt-based ATPS, and the use of ATPSs to achieve high levels of purity was studied and exhibited (Meena et al. 2015).

8.5.2 High-Resolution Techniques (Chromatographic Processes)

8.5.2.1 Ion Exchange Chromatography

Asparaginase separation using ion exchange chromatography involves the use of an anionic exchanger. Being negatively charged, asparaginase shows a high affinity toward a positively charged anionic exchanger. Ion exchange chromatography is often used in combination with other purification methods like gel filtration chromatography and affinity chromatography for enhanced efficiency and high purity. The enhanced effectiveness of combination methods was observed during the purification of asparaginase obtained from *Cladosporium* sp. using a diethylaminoethyl cellulose (DEAE cellulose) column chromatography in combination with methanol precipitation. Similar observations were also made by El-Bessoumy, who achieved 106 times high purity for asparaginase extracted from *P. aeruginosa* 50,071 using

CM Sephadex c50 chromatography in combination with ammonium sulfate precipitation (El-Bessoumy et al. 2004). DEAE (diethylaminoethyl) cellulose chromatography Q-sepharose and DEAE (diethylaminoethyl)-Toyopearl column chromatography are most frequently used as ion exchange matrices for the purification of asparaginase (Linn 2009).

8.5.2.2 Gel Filtration Chromatography

This is another high-efficiency purification technique employed commonly in enzyme purification. While being employed in the purification of asparaginase, it has reported purity levels of above 27 times with 60.8% recovery. This was observed using Sephadex G-100 gel filtration chromatography for the purification of the enzyme produced from *P. aeruginosa* 50,071 (El-Bessoumy et al. 2004). The undesired excessive dilution of the protein of interest is the primary limitation of this method. The method can be used in combination with other chromatographic methods (Mesas et al. 1990). A 98-fold purity was observed in asparaginase purified from *Corynebacterium glutamicum* using a combination of Sephacryl S-200 column gel filtration and DEAE Sephacel, while the enzyme extracted from *Cladosporium* sp. using a combination of Sepharose 6B gel filtration chromatography, DEAE-cellulose column chromatography, and methanol precipitation exhibited an 867.7-fold purity (Kumar and Manonmani 2013).

8.5.2.3 Affinity Chromatography

Affinity chromatography is a well-known method for the purification of asparaginase. A polyhistidine-tagged asparaginase enzyme obtained from *E. coli* was purified using nickel-charged affinity resins. This process reported about 86% recovery with 3.3 times purity. Affinity chromatography has also been reported for the purification of the enzyme from *Azotobacter vinelandii*. Asparaginase with above 80% purity has reported being obtained using epoxy-activated Sepharose 6 fast flow beads-based affinity chromatography. The labile nature of affinity ligands is a major disadvantage of affinity chromatography. Further, enzyme supports are expensive adding to the overall cost of the process making it commercially inviable, thus limiting its employment in the purification process (Khushoo et al. 2004).

8.6 Characterization

The biochemical properties of asparaginase vary considerably based on the source from which it has been extracted and the downstream processes involved. The biochemical properties include optimum temperature, optimum pH, and molecular weight kinetic properties stability. Tolerance to a wide range of pH and temperature, a notably substrate affinity, low Km value, and low glutamate activity are essential for the use of the enzyme in therapeutics as well as in the food industry.

8.6.1 Effect of pH on the Enzyme

pH affects both the structure of the enzyme and its charge properties. Although optimum activity was generally reported near the pH of 7, it varies considerably between different microorganisms and has been found to range from pH of 5 to that of 9.5. For example, the optimum pH for *Aspergillus niger* AK10 was found to be in the range between 6 and 11, but the maximum activity of the enzyme was found to be at pH 9 for both the crude and purified enzymes. Similarly, for the alkaliphilic microbe *S. fradiae* NEAE-82, the optimum activity was found at a pH of 8.5, while for the acidophilic *Bacillus* species, it was found at 5.0.

8.6.2 Effect of Temperature on the Enzyme

Temperature stability is an important attribute, especially for the use of the enzyme in the food industry. Asparaginase obtained from different sources exhibits not just a variation in the optimum temperature for maximum activity but a variation in the range of temperatures it can tolerate as well. For example, it was reported in a, that the enzyme underwent rapid denaturation at temperatures of 37 °C (pH was maintained at 6.8) while another study revealed that asparaginase obtained from *S. fradiae* exhibited maximum enzyme activity of 26.848 IU at 40 °C. While the enzyme obtained from some microbes is considerably thermostable, others are extremely sensitive to variations in temperatures. Asparaginase obtained from *S. brollosae* has been reported to exhibit enzyme activity between the temperature ranges from 25 °C to 60 °C, while purified enzyme obtained from *P. carotovorum* showed maximum activity at 40 °C with the activity dropping after 45 °C by up to 75% on increasing the temperature to 60 °C. Thus, knowledge about the source of the enzyme becomes important for its application. Generally, the enzyme has a tolerable temperature range between 24 °C and 45 °C.

8.6.3 Effect of Inhibitors and Metal Ions

Metal ions play an important role as electron donors and acceptors and help in the structural regulation of the enzyme. The valency of the ion impacts the activity of the enzyme. It was observed that divalent ions like Ca^{2+}, Mg^{2+}, Fe^{2+}, and $Ba2^{+}$ inhibit the enzyme activity, while monovalent ions like Na^{+} and K^{+} stimulated the activity of the enzyme in the case of asparaginase obtained from *Bacillus aryabhattai* ITBHU02. Stimulatory effects have been observed in asparaginase obtained from *Acinetobacter calcoaceticus* when subjected to Mg^{2+} and inhibitory effect in the presence of Hg^{2+}, Cu^{2+}, and p-chloromercurybenzoate (Joner 1976). Hg^{2+}, Cu^{2+}, and Ag^{2+} have been known to have a moderate inhibitory effect in the activity of the enzyme extracted from *Penicillium brevicompactum*. Similar observations showed that Fe^{3+}, Pb^{+}, and KI simulated activity in enzyme obtained from *Cladosporium* sp. While Co^{2+}, Cu+, Ca^{2+}, K^{+}, and Na^{+} inhibited it. These ions and chemicals can

be used as inhibitors. Fe^{3+} has been reported to exhibit a strong inhibitory effect in asparaginase extracted for *Bacillus subtilis*. Similarly, EDTA also strongly inhibits the activity of the enzyme extracted from *Bacillus* sp. Cu^{2+} also inhibits the enzyme activity of Asparaginase obtained from marine actinomycetes.

8.6.4 Substrate Specificity and Kinetic Parameters

K_m and V_{max} are parameters that define the specificity of the enzyme, substrate, as well as environment. Kinetic analysis of enzyme activity against substrate concentration yielded Km and V_{max} values of 0.147 mM and 35.7 IU, for asparaginase obtained from *P. aeruginosa* 50,071, while for asparaginase based on *S. fradiae* NEAE the values were 0.01007 M and 95.08 U mL^{-1}Min+, respectively. The details of these parameters for microbial asparaginase extracted from different microbes have been listed in Table 8.2.

Table 8.2 Kinetic parameters for asparaginase obtained from various microbes (modified from Cachumba et al. 2016)

Bacteria	Asparaginase activity (U mg^{-1})	$k_{M\ (mM)}$	pH	Temperature (°C)
Yersinia pseudotuberculosis Q66CJ	62.7	0.017	8.0	60
Bacillus aryabhattai ITBHU02	680.5	0.257	8.5	40
Pseudomonas fluorescens	168.4	110	8.0	37
Bacillus licheniformis RAM-8	697.1	0.014	6–10	40
Pectobacterium carotovorum MTCC 1428	35.24	657 μM	8–10	40
Nocardiopsis alba NIOT-VKMA08	158.1	0.127	8	37
Streptomyces parvulus KUA106	146	25 μM	7.5	50
Streptomyces noursei MTCC 10469	0.803	25 μM	7.5	50
Bacillus subtilis hswx88	23.8	430 μM	7.5	40
Streptomyces ginsengisoli	3.32	25 μM	7.5	30
Streptomyces thermoluteus NBRC14270	68.09	1830	8–9	63.6
Photobacterium sp.	20	760	7.0	25
Pyrococcus furiosus	550	12,000	9.0	85
Bacillus licheniformis MTCC 429	597.8	0.420	8.0	37

8.6.5 Molecular Weight

The asparaginase obtained from all bacterial sources has very similar tertiary and quaternary structures. On crystallization, they commonly exhibit a catalytically active tetramers protein form. Processing conditions can affect the tetramer structure of the enzyme. The tetrameric structure of bacterial asparaginases obtained from *E. coli* and *Erwinia* had molecular weights of 141 kDa and 138 kDa, respectively. The molecular weight of 144.42 kDa on the L-asparaginase is obtained from *P. carotovorum* MTCC 1428. The enzyme was purified by Sephadex G-100 column-based gel filtration, and the structure obtained on subjecting it to a MALDI-TOF MS analysis was observed to have a homotetramer consisting of 36.10 kDa subunits. L-asparaginase obtained from *P. aeruginosa* purified by a combination of Sephadex G-100 gel filtration and CM Sephadex C50 chromatography was found to consist of a single peptide chain having the molecular weight of 160 kDa. L-asparaginase obtained from *Pyrococcus furiosus* structurally dimers with each subunit weighing 37.86 kDa. It was found to have an overall molecular weight of 74.45 kDa. The enzyme obtained from *S. fradiae* NEAE-82 when purified by SDS-PAGE analysis consisted of subunits weighing about 56 kDa. The tetrameric structure of the enzyme plays a significant role in attributing its tumor-suppressing capacity. However, harsh processing conditions such as high pH and low temperatures (during freeze-drying) can alter the tetrameric structure of the enzyme to a monomeric structure, thus reducing its antitumor activity.

8.7 Biochemical Properties of the Asparaginase

8.7.1 Monomer Form

The monomeric form of the enzyme consists of two domains consisting of a total of about 330 amino acids. The larger N-domain and the smaller C-domain are joined by a linker of around 26 residues. The domains typically consist of an α/β fold. The larger N-terminal domain consists of an 8-stranded mixed type β-sheet while the smaller C-terminal domain is made of 4-stranded parallel β-sheets and four α-helices. A left-handed crossover exists between the β4 and β5 strands which is a distinguishing feature of asparaginase. This protein fold type is observed rarely in nature but is typically found in asparaginase and is an integral part of the activity of the enzyme. Another characteristic feature of the asparaginase enzyme is highly conserved EcAII reside Thr198 that contributes to the folding of the protein in its specific conformation. The specific Thr residue is found in the disallowed region of Ramachandran's plot although it is marked clearly in the electron density maps. Hence, it is assumed that the residue exists in a strained conformation in the natural state and is believed to exist due to the hydrogen bonds of the main chain with the amide N atoms of the adjacent residues. This Thr residue and its conserved nature is unique to the enzyme and is considered a characteristic feature of the enzyme.

8.7.2 Enzymatically Active Form

The enzyme is active in a homotetramer form. The four subunits of the homotetramer (designated as A, B, C, and D) follow a 222-crystal symmetry. Four identical noncooperative active sites exist in the enzyme at the subunit interfaces (two between subunits A and C and two between B and D). Each subunit is formed involving both the N-terminal domain and the C-terminal domain of both subunits. The binding pocket consists of the EcAII residues Thr12, Tyr25, Ser58, Gln59, Thr89, Asp90, Ala114, and Lys162 from one subunit and Asn248 and Glu283 from the other subunit. The two threonine residues are located at diametrically opposite locations (typically, above and below the CG atoms of the substrate). The active sites are structurally similar and crystallographic studies have observed that the sites consist of strictly conservative residues. It was also observed that the ligand-binding residues formed the rigid part of the active site while the flexible part of the active site controlled the access of the binding site and consisted of the highly conserved. These flexible loops in the active sites exist in different conformations based on the presence and absence of bound ligands. The confirmation of the flexible molecules is stabilized by the water molecules in the active site. They also mediate interactions between the substrate and the active site.

Asparaginase catalyzes the deamidation of asparagine to produce aspartic acid and ammonia. Asparaginase also hydrolyzes glutamine although less efficiently. L-asparaginase reacts with Nessler's reagent (K_2HgI_4) to form ammonobasic mercuric iodide ($HgO.Hg(NH_2)$). Ammonobasic mercuric iodide is colored and this is utilized in the measurement of the specific activity of the enzyme. Nessler's reagent is an endpoint indirect assay used in the determination of enzyme activity of asparaginase. In addition to these, asparaginases are also known to hydrolyze β-as particle peptide amide with considerably low yield.

8.7.3 Tumor Control Function

The enzyme can be used in the treatment of acute lymphoblastic leukemia (ALL). In a healthy human cell, asparagine is synthesized within the cell by the enzymatic conversion of aspartic acid using the enzyme asparagine synthetase which is encoded by a single gene located on chromosome 7q21.3. In the tumor cells, asparagine synthetase is not produced and hence cannot convert aspartic acid to asparagine. Thus, asparagine becomes an essential amino acid for leukemic cells, and the supply of the amino acid from the serum becomes the sole source of the amino acid to the tumor cells (Fig. 8.3).

Asparaginase is considered an effective treatment because asparaginase can hydrolyze serum asparagine to aspartic acid, thus depriving the tumor cells of asparagine essentially leading it its death while the healthy cells, which contains the asparagine synthetase enzyme, survives. Thus, the enzyme selectively eliminates the tumor cells without damaging the healthy cells. It has also been observed that the treatment with asparaginase inhibited the action of asparagine synthetase, thereby

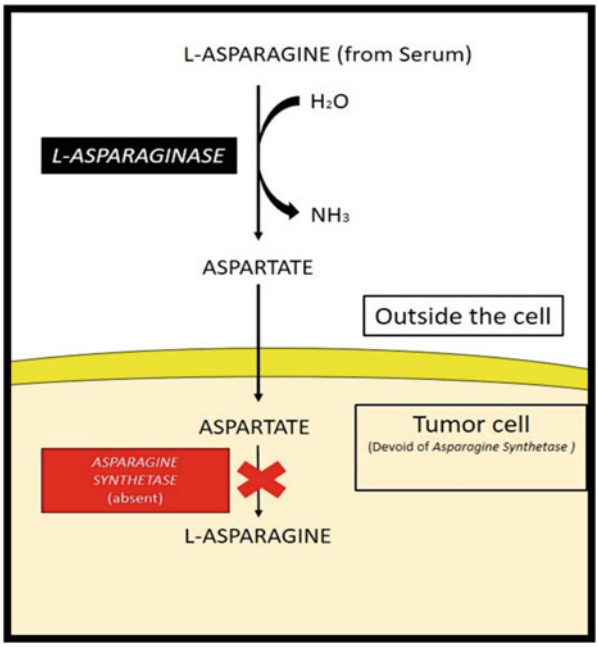

Fig. 8.3 Mechanism of action of L-asparaginase as an antineoplastic agent

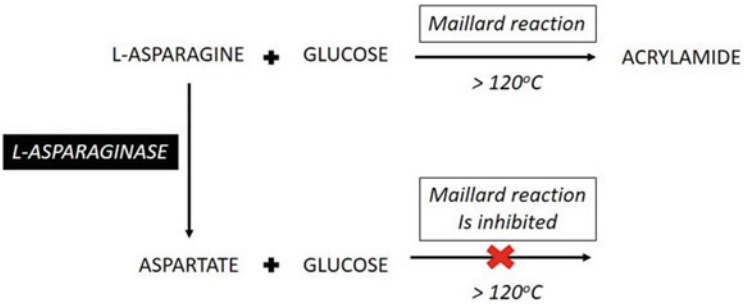

Fig. 8.4 Mechanism of prevention of acrylamide formation at high temperatures by the inhibition of Maillard reaction using L-asparaginase

limiting the production of asparagine. It also reduced metastasis without affecting the growth of the primary tumor in the case of breast cancers (Fig. 8.4).

Many infectious microbes like *Clostridium botulism* adhere to the host cell by the formation of asparaginase carbohydrate linkages. The enzymatic hydrolysis of asparaginase involves the breakdown of such asparagine carbohydrate linkages. Similarly, the cytosolic multiplication of the gram-negative *Francisella tularensis* can be prevented by the use of asparaginase as the process involves the utilization of asparagine through an arginine transporter. Hence, asparaginase is often used as the treatment of various infectious diseases. Glutamine depletion reduces the chances of survival of ovarian cell cancer. It was observed that glutamine synthetase activity is

inversely proportional to the survival of ovarian cancer cells (Furusawa et al. 2018). Since asparaginase can hydrolyze glutamine, it can be used to deplete the amount of extracellular glutamine which will assist in the cure of ovarian cancer in women. Asparaginase activity is reported to influence the lymphatic system making it capable of subduing T-cell-mediated B-cell responses thus attributing the enzyme potential therapeutic applications in treating T-cell-mediated autoimmune responses.

Another application of the enzyme is in the food industry in the control of acrylamide. Acrylamide is a known carcinogen and is also neurotoxic. Acrylamide formation in foods occurs during the browning process at temperatures greater than 120 °C when asparagine reacts with the reduced sugars. The reaction is known as the Maillard reaction. Acrylamide formation is a major concern in a variety of oven-cooked and fried foods. Asparaginase interrupts the interaction of asparagine and sugars avoiding the acrylamide formation. A simple pretreatment before cooking the food limits the acrylamide content to safe levels and renders it safe for consumption.

8.8 Mechanism of Action of the Asparaginase

Asparaginase is the enzyme that is responsible for the hydrolysis of asparagine to aspartic acid and ammonia. The enzymatic hydrolysis occurs in two steps and involves the formation of the intermediate beta-acyl-enzyme. Initially, the enzyme's nucleophile residue is activated in the presence of a strong base (Fig. 8.5). The activated residue attacks the amide carbon atom of asparagine, the substrate. This results in the formation of the beta-acyl-enzyme intermediate. The second step of the reaction involves the attack of ester carbon by a water-activated nucleophile.

Asparaginase can also hydrolyze glutamine but the efficiency is significantly low. For instance, asparaginase obtained from *Serratia marcescens* was reported to

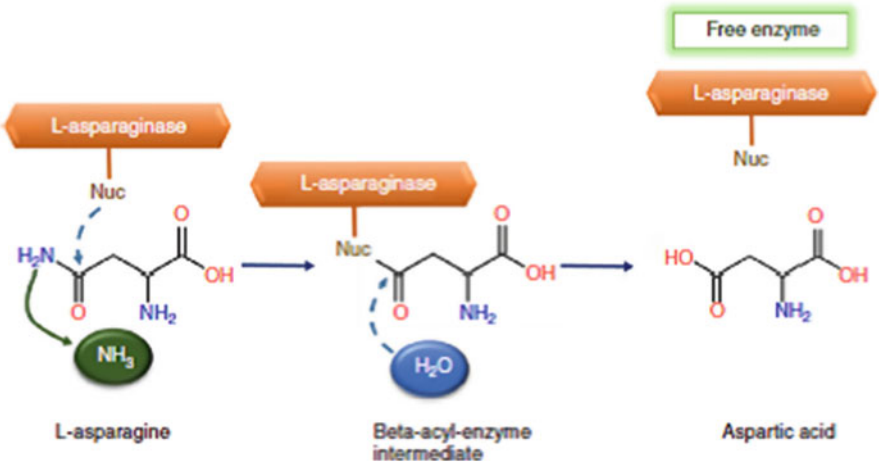

Fig. 8.5 General mechanism of catalysis reaction of asparaginase. (Source: Cachumba et al. 2016)

hydrolyze about 5% of L-glutamine when compared to asparagine hydrolysis. Glutamine functions as a competitive inhibitor to asparagine in the asparaginase-based hydrolysis reaction. The structural similarity of the two amino acids is responsible for the similarity in their hydrolysis processes too.

8.9 Biotechnological Potentials of Asparaginase

Asparaginase finds application in multiple industries, including therapeutics. Its potential for use in the treatment of various cancers has spawned much research and developments with regard to its production, purification, optimization, activity, and chemical characteristics. The use of the enzyme is currently limited in therapeutics because of the following challenges.

8.9.1 Varying Pharmacokinetic Properties

The enzyme is extracted from different sources by the application of different purification techniques. This is because of the lack of a well-optimized source and process for the specific application. As a result, it has been observed that although the enzymes exhibit a similar mechanism of action in the serum, the pharmacokinetics is different making it difficult to define the process accurately.

Recombinant DNA technology enables the effective production of the enzyme from diverse sources by microbial fermentation at an industrial scale. Asparaginase enzyme with cytotoxicity comparable to that extracted from *Escherichia coli* was extracted from *Zymomonas mobilis* (Einsfeldt et al. 2016). *Streptomyces griseus* belonging to the marine actinobacterium phylum was reported to have the ansA gene. This gene when expressed in the *E. coli* M15 resulted in a threefold increase in enzyme production (Meena et al. 2015). Similarly, an artificially engineered gene containing an enterokinase cleavage site and a His tag at the N-terminal was expressed in *E. coli*. The synthetic gene was engineered to have codons that matched the ones used by *E. coli*. The genetically modified *E. coli* expressed the enzyme intracellularly with a yield of 3.6 IU/mL as well as extracellularly with a yield of 0.13 IU/mL. The enzyme secreted extracellularly was also observed to have inhibitory effects on cancer cells. Leukemia cell proliferation was also reported to be inhibited by recombinant asparaginase II extracted from a thermotolerant variant of the *E. coli* isolated from camel dung. As the secretion of the enzyme makes downstream processing easier and economical, most of the recombinant genes are engineered to contain a secretory signal which may either be the native signal of the organism or any other desired signal sequence that fits the frame of the asparaginase gene. Recombinant *E. coli* cloned with the genes containing the secretory signal sequence has been reported to secrete the enzyme into the culture medium at optimum conditions. Further, the use of response surface methodology to optimize the components provided a net yield of about 17.386 U/L of the pure enzyme (Ghoshoon et al. 2015).

The signal sequences and promoters can be modified for enhanced enzyme production. The use of combinations of random mutagenesis and different signal sequence and promoter combinations has been reported to be used to achieve a step-by-step increase in yield of asparaginase from recombinant *Bacillus subtilis*.

8.9.2 Low Substrate Specificity

Asparaginase can also hydrolyze glutamine which acts as a competitive inhibitor of asparagine. A high glutaminase activity can result in low protein synthesis in the cell. This can cause adverse effects such as liver dysfunction pancreatitis, neurological seizures, intracranial hemorrhage, and leucopenia.

Asparaginase with improved half-life and thermal tolerance, free of glutaminase activity, was developed and extracted from *Bacillus licheniformis* through mutations (Sudhir et al. 2014). The mutant D103V showed a threefold increase in the half-life and thermal tolerance, compared to the protein extracted from the native wild organism. The mutant was also observed to exhibit enhanced substrate affinity with a K_m value of 0.42 mM and V_{max} of 2778.9 mol min^{-1}.

Hypersensitivity reactions, immunogenic complications, and side effects: Hypersensitivity reactions have been observed in 30% of the patients who have been administered the enzyme. The various complications associated with the asparaginase hypersensitivity include mild to severe reactions, urticaria, bronchospasm, serum sickness, hypotension, anaphylaxis, and immunosuppression. Asparaginase is substantially immunogenic and exhibits significant clinical reactivity in the hosts. The reaction may manifest as silent inactivation or systematic anaphylaxis. Antibodies may be generated by the immune system and the enzyme can be deactivated, rendering the therapy useless. Side effects like hepatotoxicity, hyperglycemia, pancreatitis, and dyslipidemia have been observed in patients which are due to immunogenic complications. *E. coli* enzyme that is resistant to the lysosomal proteases by employing rational design methods utilizing the tools like genetic algorithms and molecular dynamics was developed (Patel et al. 2009). The cleavage start site of asparagine endopeptidase (AEP), a lysosomal protease, was identified as N24 and was replaced with a mutant protein N24G. N24G had superior resistance to proteolytic cleavage but was inferior in terms of enzyme activity. This was later further modified by the replacement of alanine at the same position (N24A) resulting in an enzyme with higher activity due to a unique hydrogen bonding network. Later, this enzyme was further modified for therapeutic applications specifically by the addition of a mutation in the interface position 195 constructing a double mutant N24A R195S reducing its glutaminase activity thus enhancing specificity and reducing side effects. Compared to the native asparaginase II extracted from wild-type *E. coli* W66Y and Y176F, the mutated variant of the enzyme was more specific with reduced glutamerase activity while also being effective against ALL cells (Mehta et al. 2014). Similarly, mutations induced in the immune-dominant epitopes K288S/Y176F rendered the enzyme decreased immunogenicity by about ten times, while also reducing its antigenicity. Epitope

mapping also allows the identification and eliminating of the T-cell epitopes and class II MHC agretope. This reduces the immunogenicity of the enzyme to a great extent. Thus, a detailed analysis of the physicochemical parameters of the epitopes can be used to engineer proteins with reduced immune response. The therapeutic protein thus engineered to reduce the immunogenicity will have a higher retention time in circulation.

8.9.3 Asparaginase Resistance

Apart from immunogenic inactivation of the enzyme, resistance may be exhibited by the patient primarily due to the derepression of the asparagine synthetase gene. This may occur due to differences in the gene expression profiles and genetic background, which have led to a cellular and biochemical relationship in the microbial asparaginase resistance mechanism. This can be solved by altering the structure of the enzyme to inhibit the action of specific cellular mechanisms on the enzyme. Recombinant enzyme extracted from *Wolinella succinogenes* having V23Q and K24T mutant amino acid substitutions and an N-terminal peptide which binds to heparin-based trypsinolysis resistance has high efficiency in therapeutic applications. (Sannikova et al. 2016).

8.9.4 Expression as Fusion Proteins

The fusion of the target protein with an additional partner protein to produce a complex protein is a procedure employed in pharmaceutical industries for the commercial production of therapeutic drugs. Genetic fusions are employed primarily to extend protein half-life. Fusion of the protein with Fc antibody and albumin enhances its in vivo stability and immunogenicity. The elimination of the hydrophobic amino acids selectively from the attached polypeptides can also decrease immunogenic response fusing target proteins with inert peptide repeat polymers is possible and attributes advantages such as easier manipulation of *E. coli* system, lower production costs, and efficient purification of the enzyme. Fusion of the recombinant unstructured polypeptide XTEN to the protein can improve its plasma half-life.

8.9.5 Advanced Delivery Systems for Asparaginase Delivery

The drug delivery route is often considered to be as important as the drug development process because the route of drug delivery determines the rate of release of drug to the bloodstream, dosage of the drug to be administered, and controllable level of the drug. Efficient delivery systems can help in the control of several undesired side effects and hypersensitivity disorders. The pharmacokinetic properties and circulatory half-life of the drug are dependent on the drug delivery system and play an important role in regulating the efficiency of the therapeutic protein. Drug carrier

molecules can be used to control the timing of drug action, improve the circulatory half-life, as well as enhance specificity. One such approach included the use of RBCs as drug delivery systems. The enzyme can be entrapped within the erythrocytes to protect them from the immune system. This will also extend the half-life of the enzyme to be equal to that of the erythrocytes (Domenech et al. 2011). Additionally, since the ABO and Rh blood group compatibility is also considered during the construction of the delivery vehicle molecule, the probability of rejection can also be controlled (Agrawal et al. 2012). Nanoparticles and nanocarriers such as emulsion polymerization, cross-linked nanogel matrices, dendrimers, and carbon nanotubes can also be used as carriers to deliver the enzymes at specific locations. Asparaginases can also be delivered by entrapping them in liposomes without losing their activity and anticancer potentiality. The hydrophobic part of the protein can be entrapped in one of the lipid bilayers while the hydrophilic moieties can be carried in the inner aqueous phase. The thin layer hydration method has been used to successfully trap the enzyme using soya bean lecithin and cholesterol. Cholesterol was used to retain the stability of liposomal particles (De and Venkatesh 2012).

Biotechnology can thus be potentially applied in the development of several aspects and industries about asparaginase from the selection and recombination of a high yielding source organism to the development of a targeted delivery system for the efficient therapeutic application of enzyme. Having a deep understanding of the fundamentals of recombinant DNA technology and the various protein engineering strategies available helps in the characterization of the different variants of the enzyme from diverse sources and to manipulate them according to our interests and requirements. An overview of the recent research outcomes and developments in the biochemical industrial sector portray that various applications of biotechnology in tailoring various techniques and approaches for increased production and inducing the desired quality is asparaginase.

8.10 Conclusion and Future Prospects

The molecular analysis of various asparaginases has made a deeper understanding of the molecular mechanics of the enzyme, structure-function relationship. Similarly, the growth of biotechnology has provided the opportunity to develop more viable sources of the enzyme, enhance the properties of the enzyme, and modify the physicochemical structure as well as biochemical characteristics of the enzyme. Asparaginase genes from microbial sources such as *E. coli*, *B. subtilis*, *N. Alba*, *Pseudomonas fluorescens*, *and Yersinia pseudo tuberculosis* have already been extracted, cloned, and recombined for increased expression. Research is underway for the discovery of methods to exploit the anticancer properties of the drug in therapeutics as a cure for ovarian, lung, and breast cancers. The potential use of the enzyme for the treatment of various infectious diseases is also being studied. Another major field of research is efficient systematic methods to modify the biochemical properties of the drug to better suit them for therapeutic applications. This includes methods to reduce side effects and undesired immunological responses

and resistance to the drug. The development of protein engineering will help in the development of such methods. Various enzyme production optimization methods ranging from discovery and designing of genetically engineered recombinant source organisms to the development of novel fermentation techniques and advanced downstream processing techniques are being studied. It is expected that a viable and standard enzyme production protocol will be developed shortly. This would enhance the global production and application of the enzyme. The divergence in the applications of the enzyme is also predicted. Currently, the enzyme is used in the food industry apart from medical applications. The asparaginase formulation converts the asparagine in raw foods to aspartate thus controlling acrylamide formation by up to 97% without affecting the taste, appearance, or palatability of the final product. The enzyme can be potentially used to develop biosensors and this is being studied. The enzyme holds considerable potentials, and various methods to apply them in different industries as well as the discovery and development of new properties and applications are expected to happen in the future.

Acknowledgments Dr. Surajbhan Sevda thanks NIT Warangal for the research seed grant (P1128) for support of this work.

Conflicts of Interest There is no conflict of interest to declare.

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Laccases: Production, Harvest, Recovery, and Potential Industrial Application

9

Ritu Bansal and Surajbhan Sevda

Abstract

The laccase enzymes are produced by various insects, bacteria, fungi, and plants. They come under the group of copper-containing oxidase. These enzymes are produced with both submerged and solid-state fermentation processes. The biological biomass waste material can also be used to produce laccase enzyme using solid-state fermentation. Laccases are mostly used in different industries, such as food, paper, and pulp, pharmaceutical, and textile. In this book chapter, various aspects of laccase, such as structure, production in bioreactors, downstream processing, factors affecting the laccase synthesis, and regulation of this enzyme, are discussed in details.

Keywords

Laccases · Production · Harvest · Recovery · Characterization

Abbreviations

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AREs	Agent reaction components
DABSA	2,5-diaminobenzenesulfonic corrosive
DMP	Dimethoxyphenol
EDTA	Ethylenediaminetetraacetic acid
HSEs	Heat stun reaction components

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_9

IET	Inward electron move
kDa	Kilodalton
LMS	Laccase-interceded system
MREs	Metal reaction components
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SmF	Submerged fermentation
SSF	Solid-state fermentation
XREs	Xenobiotic reaction components

9.1 Introduction

Laccases (EC 1.10.3.2, benzenediol:oxygen oxidoreductases) are commonly extracellular monomeric glycoproteins with the position in the multicopper oxidase family (Mate and Alcalde 2017; Agrawal et al. 2020a). It has the property to catalyze the oxidation of a varied scope of compounds related to the 4 e^- declines of subatomic oxygen to water (Agrawal and Verma 2019a). They have a lower redox potential (450–800 mV) as compared to ligninolytic peroxidases (less than 1 V) (Baldrian 2006). It shows an exceptionally moderated three-dimensional design, including four substrates binding loops and four profoundly rationed conserved sequences with amino acids behaving as copper ligands (Monographs 2020). The first laccase was accounted for by Bertrand, who said laccase was liable for the shading change in mushrooms of the *Boletus* class when in contact with air. An enormous number of organisms have been affirmed as laccase makers, with white-rot fungi being the most perceived (Verma and Madamwar 2002a, b, c).

Among fungi species, the basidiomycetes, mainly *Agaricus bisporus, Pleurotus ostreatus, Trametes versicolor,* and *Phanerochaete chrysosporium*, produce different laccase isoforms (Arregui et al. 2019; Agrawal et al. 2018; Verma and Madamwar 2005).

Laccases have extensive substrate specificity with phenols, polyphenols, aromatic amines, thiols, inorganic ions, and heterocyclic compounds. They are brilliant catalysts for industrial progressions, as they are comparatively vigorous and utilized in the company of numerous organic solvents. Because of their unspecific catalytic action, they tend to have high latent for detoxification and decolorization of industrial runoffs, polluted with synthetic dyes of varied biochemical structures (Agrawal and Verma 2020a).

9.2 Structure of Laccase

It exists in different structures, primarily monomeric yet additionally present in some homodimeric, heterodimeric, and multimeric structures. Its atomic mass spans from 50 to140 kDa, contingent on its source. Laccases as multi-copper oxidases (MCOs) have four copper particles in amazingly extraordinary oxidation states: one type 1, one type 2, and two type 3 s, all forming their catalytic site. Basic laccases have three homologous cupredoxin areas. Their mononuclear copper site occurs in area 3, and their trinuclear bunch is shaped at the interface between space 1 and 3. The role of domain 2 is to join and position areas 1 and 3, empowering the arrangement of the trinuclear group (Agrawal et al. 2020b). Conversely, in two-domain laccases, obtained from bacteria, their mononuclear copper site exists in domain 1 or 2, yet, for the arrangement of their trinuclear bunch, they need to oligomerize as homotrimers, which produces a catalytic site at the interface between the domain 1 of one monomer and the domain 2 of the other monomer (Fig. 9.1). Laccases are labeled as "moonlighting" proteins, attributable to their various biological activities (Arregui et al. 2019).

The organization of the copper particles is generally monitored amid laccases (Fig. 9.1). The T1 Cu includes two histidine nitrogen iotas and cysteine sulfur, what's more, and is described by a brilliant S (π)! Cu (dx 2 − y 2) charge move assimilation band at about 600 nm, ε600 nm > 3000/M/cm answerable for the extraordinary blue tone of chemicals. The T2 copper site, deliberately situated near the T3 binuclear copper focus, is organized by two histidine buildups and a water atom, while each T3 copper is facilitated by three histidines and a spanning ligand, for example, hydroxyl moiety, which shows retention in close UV, at $\lambda_{max} = 30$ nm.

The mononuclear T1 Cu site connects to trinuclear group T2/T3 through the exceptionally saved HCH theme, where the cysteine in T1 restricting Cu transports e^- to every one of the two histidines that arrange T3 copper particles.

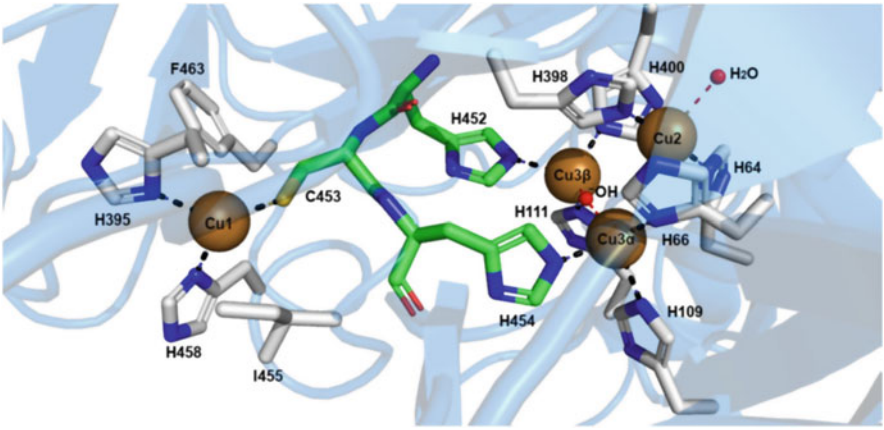

Fig. 9.1 The laccase structure. (Source: Arregui et al. 2019)

When all is said and done, laccases exhibit a wide restricting pocket covered between domain 1 and 3 and near T1 Cu focus, fundamentally settled by hydrophobic collaborations. In *B. subtilis* CotA laccase, the T1 Cu center is located at the lower portion of the substrate limiting region and is introduced to the dissolvable, which works together with the substrate particles by the imidazole ring of one histidine ligands. Still, in the remainder of three space bacterial laccases whose design are represented, the T1 site is hindered by a couple of discretionary plan segments, with vacillated length, blend likewise, structure. The little substrate limiting pocket of these proteins has all the earmarks of being according to their higher explicitness to minimal metal particles Cu + and Fe^{2+}, exhibiting that these laccases should continue fantastically as metallic oxides in their nearby microorganisms with a task in copper impediment. Regardless, even at diminished capability, these proteins are furthermore prepared to oxidize phenolic and non-phenolic substrates; additionally, they can also have extra limiting pockets to oblige heavier substrates (Monographs 2020).

9.3 Laccase Production and Purification

9.3.1 Laccase Production in Bioreactor

Laccase production is highest for *Basidiomycetes* (white-rot fungi). Initially, submerged fermentation has been used for production. It results in a homogeneous distribution of nutrients, resulting in full contact and absorption of nutrients by cultured microorganisms. In correlation with SF, SSF all the more intently reproduces the microorganism's indigenous habitat and has various points of interest such similar to a more straightforward strategy, having lower energy utilization and less contamination just as higher item recuperation.

The examples in the type of water, soil, and rocks were gathered from different spots of Tattapani hot spring, Chhattisgarh, India. The samples (temp = 92 °C, pH 8.4) were saved at 4 °C till additional utilization. Dilutions with sterile water were done then, and the examples were enhanced on supplement stock by hatching at 55 °C for 24 h. They were spread onto supplement agar medium and brooded at 55 °C for 24 h. Because of morphology and color, distinctive colonies of bacteria were streaked independently and purified for some time.

The thermophile bacterial separates were evaluated for laccase movement through a straightforward plate examination, utilizing guaiacol (5 mM) as a substrate. The colonies were spotted on supplement agar plates enhanced with guaiacol and incubated at 37 °C for 48 h. The presence of rosy earthy-colored tones demonstrates the laccase delivering capacity of the separated species. For affirmation, similar bacterial strains were screened once more, utilizing DMP (dimethoxyphenol) as a substrate. During the test, 1% DMP was blended in with agar and filled with the sterile petri plate. After hardening, the roundabout wells were readied also, loaded up with 100 mL of unrefined laccase, and hatched for 24 h at

50 °C. The development of the orange shading zone affirms the presence of laccase protein.

The identification procedure has three steps: extraction of genomic DNA, PCR (polymerase chain reaction) amplification, and gene sequence analysis. DNA was extracted from bacterial cells according to Marmur's method (Siroosi et al. 2016).

Supplement stock (50 mL) enhanced with 2 mM $CuSO_4$. $5H_2O$ was vaccinated with 500 mL of an overnight culture of disconnect for lowered creation of laccase. The culture was kept up at 55 °C (pH = 7) for 36 h at 120 rpm. Aliquots from aging stock were taken and centrifuged for 10 min at 8000 rpm. The supernatant containing laccase was considered as an extracellular compound and tested utilizing ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as a substrate (Sharma et al. 2019). Then again, the intracellular laccase movement was too assessed. For this, the stock was centrifuged and gotten pellet was suspended in 3 mL of 100 mM phosphate cushion (pH 7.0). At that point, sonication was directed at 50% sufficiency for 5 min at 1 s/cycle, utilizing a test sonicator to extricate the intracellular proteins. To eliminate the cell parts, the suspension was centrifuged at 8000 rpm for 20 min at 4 °C, and the supernatant was considered as intracellular protein. The ammonium sulfate precipitation and particle trade chromatography were performed for the cleaning of extracellular unrefined laccase. The ammonium sulfate precipitation was performed at the time frames, 20–40%, 40–60%, 60–80%, and 80–100%, utilizing the standard table announced somewhere else (Wingfield 1998).

The example with the greatest precipitation was centrifuged for 15 min at 10,000 rpm. The pellet was at that point resuspended in sodium phosphate support of 50 mM (pH 6.0) and was dialyzed against the sodium phosphate support of 20 mM (pH 6.0). Particle trade chromatography was performed to additionally filter the example. DEAE cellulose was utilized as a fixed stage in a section with a bed volume = 20 mL. At first, the column was equilibrated, utilizing a sodium phosphate cradle of 50 mM at pH 6. An example volume of 1 mL was included in the segment and eluted utilizing inclination NaCl as a portable stage. Various parts were gathered and examined for laccase action. The division that has the greatest movement was pooled. Laccase movement was resolved at every sanitization step, utilizing ABTS as a substrate (Galhaup et al. 2002). The combination containing 300 mL of the compound and 0.5 mM ABTS (last fixation) in CH_3COONa derivation cradle of 100 mM at pH 4.5 was hatched at 60 °C for 10 min. From that point forward, 0.5 mL of 80% TCA acid was added to hinder the response. The oxidation of ABTS was controlled by checking the absorbance increment at 420 nm. The measure of chemical required to oxidize 1.0 mmol of ABTS/min is measured as 1 unit of chemical. The concentration of aggregate protein was measured by Lowry's strategy, utilizing UV-Vis spectrophotometer at a frequency = 620 nm (Lowry et al. 1951).

9.3.2 Factors Affecting Laccase Yield

Laccase movement has vanished after a specific figure of response cycles. Let's say, within the sight of free revolutionaries created by $1e^{-}$ deduction of phenols, sweet-smelling amines, or go-between, the catalyst has been accounted for two at last inactivate. Albeit the instrument of inactivation by free extremists isn't surely known, it might include oxidation of significant deposits close to the dynamic site that are fundamental for looking after protein structure, as exhibited for different compounds catalyzing comparative responses. Another significant issue is the movement and steadiness within the sight of natural solvents; because of the hydrophobic idea of most substrates, the presence of cosolvents is important to build substrate accessibility. Most compounds are vulnerable to movement misfortune within the sight of natural solvents, attributable to denaturation and different systems. They are not excluded, given their precariousness within the sight of methanol, ethanol, $(CH_3)_2CO$, and acetonitrile, just like varied water-dissolvable solvents (Arregui et al. 2019).

9.3.3 Regulation of Laccase Synthesis

Laccase action has likewise been demonstrated as reliant on fixation and type of carbon and nitrogen sources just as on their proportion. Nitrogen source assumes a vital part in laccase creation, with impacts relying upon its temperament and fixation in culture media. Change in laccase action in light of nitrogen focus is a disputable issue since instances of action increments have been portrayed under both restricting and non-restricting conditions (Janusz et al. 2007; Xiao et al. 2003). For the most part, inorganic nitrogen sources bring about low degrees of laccase with adequate biomass creation, while natural nitrogen sources give high laccase harvest with great parasitic development. Yeast extricate is a standout among other nitrogen sources that build the products of laccase compounds (Piscitelli et al. 2011; Eggert et al. 1996). The protein yield is likewise expanded through the addition of the medium with an extra nitrogen source, such as amino corrosive L-asparagine.

Following the effective screening of laccase-creating local hosts, laccase creation is improved by aging innovation advancement as for maturation type, medium arrangement, and development boundaries. Improving laccase yields is fundamental for lower creation costs and advanced mechanical uses of the chemical, which depends on comprehension of laccase articulation guidelines. Various distributions and audits have been dedicated to articulation guidelines of laccases (Iracheta-Cárdenas et al. 2016). Articulation of laccase isozyme qualities is differentially managed all through aging and in light of medium organization, such as metal particles and xenobiotics, just as supplement types and levels. Laccase articulation examination has been performed on mRNA and protein levels, and the unmistakable reactions of species strain just as qualities no uncertainty portray laccase articulation guideline. In agreement, different cis-acting responsive components have been recognized in laccase advertiser locales, for example, metal reaction components

(MREs) (Janusz et al. 2007; Xiao et al. 2003), ACE1 copper-responsive record factor restricting destinations (ACE1), xenobiotic reaction components (XREs), cancer prevention agent reaction components (AREs), heat stun reaction components (HSEs), CreA restricting destinations (CreA), and NIT2 restricting destinations (NIT2) (Janusz et al. 2007; Piscitelli et al. 2011). In any case, the capacity of the greater part of the putative responsive components isn't tentatively approved, and how they interface with record factors stays slippery (Legerská et al. 2016; Wikee et al. 2019; Batista-García et al. 2016; Zerva et al. 2019). Copper particles are presumably the most utilized inducer in laccase creation, and the ACE1 restricting site addresses the most surely known administrative component in the laccase advertiser locale. In yeast, ACE1 and CUF1 have tradable N-terminal copper-clenched hand DNA restricting themes regardless of inverse parts in keeping up copper homeostasis. Then again, a copper-responsive laccase quality without a standard ACE1 restricting site inside its advertiser may be controlled through a nonconventional copper-responsive component or an alternate system (Janusz et al. 2007; Xiao et al. 2003; Piscitelli et al. 2011). In any event, when copper particles can't actuate laccase creation, they balance out the copper-containing synergist focus of the catalyst, in this way adding to laccase action. Other than copper, manganese and zinc are additionally regularly found to animate laccase combination. Writing depicting laccase enlistment by xenobiotics, e.g., lignin breakdown items, dyestuffs, and natural poisons, has been collected. Combinational acceptance of laccase creation by metal particles and natural mixtures can be either synergistic or hostile coculture of laccase-creating strains with different microorganisms is a characteristic method to incite laccase creation, as either yield increment or acceptance of new isozymes, and can be more successful than compound enlistment (Fan et al. 2014). Microbial cooperation with laccase inciting impacts differ with the strain, yet the design of initiating metabolites and the actuating system remain to a great extent obscure phenolics and lysing compounds delivered by restricting organisms have likewise been proposed to have laccase-prompting capacity (Jeon et al. 2012; Fang et al. 2011). Supplement types and fixations have been broadly concentrated with regard to contagious development and chemical discharge. Since the basidiomycetes show a wide variety in their reactions, no speculation can be made on the best carbon and nitrogen sources or their ideal fixations. Lignocellulosic squanders containing starches and inducers are frequently added, bringing about advantages, for example, lower creation costs, squander reuse, and laccase creation upgrade (Cañas and Camarero 2010; Yang et al. 2017). In numerous organisms, laccase is created by optional digestion; that is, laccase union is enacted via carbon or nitrogen exhaustion. This no uncertainty requires a long creation cycle and hampers the mechanical creation of laccase. Subsequently, a promising business laccase maker should create laccase with significant returns and a short maturation cycle (Arregui et al. 2019).

9.3.4 Purification and Characterization of Laccase

Purification of laccase is done by a series of methods: ammonium sulfate precipitation, ion exchange chromatography, gel filtration chromatography, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Agrawal et al. 2019; Agrawal and Verma 2020b).

Ideal temperature and pH were controlled by performing enzymatic tests at various temperatures (20–80 °C) and pH 3–8 separately. The pH level was changed, utilizing the accompanying cushions: 0.1 M citrate support at pH 3–5, 0.1 M phosphate cradle at pH 6–8, and 0.1 M carbonate cradle at pH 9. The steadiness of the decontaminated laccase at different temperatures was researched by preincubation of the filtered laccase at various temperatures somewhere in the range of 4–70 °C for 1 h, trailed by the assurance of the lingering action (Telke et al. 2009). The impact of pH on laccase dependability was dictated by hatching the decontaminated protein at 4 °C in various pH levels for 24 h and deciding the remaining action. Substrate focus scopes of 50–500 l M, 500–1500 l M, and 1000–2500 l M were utilized for motor investigations against ABTS and DMP separately. The impacts of metal particles, including Hg^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, and Ba^{2+}, and inhibitors, such as EDTA (ethylenediaminetetraacetic acid), NaN_3, DTT, and SDS, on laccase action were explored by joining them in to test combination before the assurance of leftover movement (More et al. 2011; Lu et al. 2007; Kiiskinen et al. 2002).

Kinetic study of laccase-catalyzed oxidation of ABTS at various concentrations (10–200 μM) was done at pH 4.5 and 25 °C to decide kinetic constants, V_{max} and K_m. The kinetic constants were determined from the Lineweaver-Burk plot of the complementary of initial velocities and substrate concentration (Ezike et al. 2020).

9.3.5 Biochemical Properties of Laccase

Laccases are mostly monomeric, dimeric, and tetrameric glycoprotein. Glycosylation assumes a significant part in copper maintenance, warm steadiness, weakness to proteolytic debasement, and discharge (Yaropolov et al. 1994). Upon sanitization, laccase proteins exhibit impressive heterogeneity. Glycosylation substance and piece of glycoprotein differ with development medium arrangement (More et al. 2011).

9.3.6 Mechanism of Action of Laccases

The general laccase response includes $1e^-$, successive oxidations of four particles of diminishing substrates, simultaneously with two twofold e^- diminishings of oxygen atoms into their separate water atoms (Fig. 9.2). This cycle is joined by a reactant trade of 4 H+ counterparts. From the underlying, unthinking, and kinetical perspectives, a laccase response is drawn closer as two half-responses associated

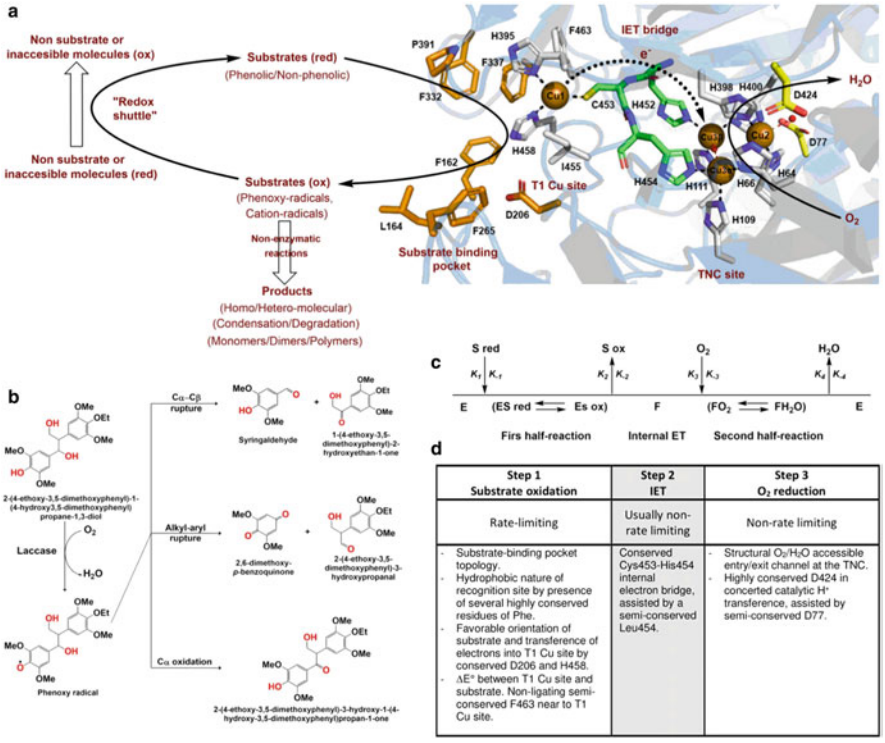

Fig. 9.2 Shows the mechanism, structural elements, and kinetic model for laccase reaction and function properties. (**a**): Shows the action mechanism in the active site of laccase. (**b**): Shows the laccase machismo on a lignin model. (**c**): Shows the ping-pong model for laccase reaction. (**d**): Shows the functional and structural element in various steps of laccase reactions. (Source: Arregui et al. 2019)

by an inward electron move (IET) step, helped by the reactant copper particles situated at the T1 Cu and T2 Cu/T3 Cuα/T3 Cuβ trinuclear cluster sites (Fig. 9.2) (Polyakov et al. 2017).

Notwithstanding developments in the comprehension of activity of laccases regarding assembly–work, total representation connecting their atomic properties in addition to systems with their motor exhibition be indistinct (Fig. 9.2). This state can be perceived as dependent on the developmentally changed expansive scope of natural particles fit to be oxidized by a laccase and its family member capacity of different laccase to grind substrates into acknowledgment locales as well as position them to be oxidized, restricting a coordinated plan (Fig. 9.2).

9.3.7 Biotechnological Potentials of Laccase

There are over 15 laccase-grounded business items used in the material, food, and paper industry that reflect the possibility of their mechanical use. The best and adaptable applications have been made for material business, particularly for the whitening of indigo-stained denim, for instance, Denilitel, DeniliteITM, Zylite, and Bleach-cut 3S. Additionally, commercialization of the laccase MetZyme, which has high ligninolytic properties, opens the opportunity for business utilization of further laccases soon (Rodríguez-Couto 2019). Yet, no business laccase things for water treatment are available yet. Taking everything into account, a few tests clearly represent the laccase's suitability to wipe out harms in certified wastewater. *Trametes versicolor* form of laccase has shown to be viable at diminishing estrogen obsessions from a city wastewater treatment plant at Rolla, Missouri (USA), attempted at lab scale. Considering cost practicality, laccases may present huge inclinations over other adulterating proteins in city wastewater treatment. The infectious crude removes from *T. pubescens* MUT 2400 changed target particles and reduced to ~70% of the hidden fixation of 2-hydroxybiphenyl, Naproxen, DF, and diethyl phthalate under real city wastewater conditions in northwestern Italy (Turin). Also, the helpful result of killing phenol from liquid treatment office tests was displayed using SP504 laccase from Novozymes (Franklinton, NC, USA) (Steevensz et al. 2009).

9.4 Laccase Applications

In the last 10 years, there has been increased biotechnological applications of laccases. Laccase-induced oxidations result in the rise of radical species, which advances toward artificial and degradation procedures.

Revolutionary destiny can trail three distinct ways reliant on numerous elements: (1) Oxidative coupling is the chief instrument prompting homo- or cross-coupling of particles into dimers or conceivably in like manner polymeric types. This course deciphers in vitro into biotechnological applications in shading and polymer mix and joining for surface functionalization. Then again, (2) dependent upon their security and redox reversibility, revolutionaries might go through change, yielding stalemate things; (3) it may go probably as middle individuals for extra oxidation of non-phenolic compound causing security cleavage. The last two kinds of laccase exercises can be recreated in vitro into biotechnological applications, for instance, blurring advancement and bioremediation (Agrawal and Verma 2019b, 2020c).

9.4.1 Textile Industry

Laccases have a wide range of applications in the textile industry due to their properties of fiber bleaching and dyeing and act as a vital "green tool" to adjust and enhance textile characteristics.

9.4.1.1 Fiber Bleaching

Laccases have arisen as a fiber-saving option in contrast to the most generally utilized dyeing specialist, hydrogen peroxide. At the point when joined through H_2O_2 created by glucose oxidase, laccase treatment of cloth texture came about into successful whiteness increment and alteration of sum and sort of lignin surface practical gatherings.

The cycle, solidifying hydrogen peroxide with laccase-interceded system (LMS) pretreatment allowed a lower estimation of hydrogen peroxide, diminished blurring temperature, and abridged biting the dust time. By oxidizing indigo tone on denim pieces of clothing into uncolored combinations, laccase treatment has allowed procuring the ideal wash light effect on blue denim pants without using remorseless oxidative subject matter experts, for instance, sodium hypochlorite. The use of laccases for denim arrangement returns to 1996 when the essential business laccase status DeniliteITM was dispatched accessible by Novozymes. From that point forward, an adjusted meaning of laccases has been made to target unequivocal garment's wet cycle conditions, as significantly overviewed by Rodriguez-Couto. Solis-Olba used a LMS reliant on ABTS to denim decoloration, watching that denim strands were not hurt during the treatment and the natural impact of the collaboration was diminished as the remaining water of denim decoloration is biodegraded (Pezzella et al. 2015).

9.4.1.2 Textile Dyeing

The utilization of laccases to incorporate colorants in situ has emerged as a "green" route for material coloring at mellow interaction conditions. Laccase-interceded coupling and joining responses is utilized for shading different materials. In the main endeavor of cellulose material coloring (Hadzhiyska and colleagues), cotton cellulose was colored in situ by polymeric color created by oxidative coupling of lackluster 2,5-diaminobenzenesulfonic corrosive (DABSA) and 1-hydroxyphenyl (catechol) with laccase-treated filaments, acquiring up to 70% of color obsession.

Laccase-produced polymers offered to ascend to new shading states from dull earthy colored to green-yellow and supplanted color in the overdyeing interaction. By this interaction, overdyeing of denim was effectively accomplished with satisfactory stages regarding solidness. Likewise, cowhide was shaded by laccase activity utilizing dihydroxynaphthalenes as substrates.

9.4.2 Baking Industry

Laccase capacity to cross-connect arabinoxylan lattice in batter by dimerization of esterified ferulic corrosive produces expanded strength, solidness, and decreased tenacity of batter, and accordingly coming about into its improved machinability. Furthermore, expanded volume and improved piece design and non-abrasiveness of sponsored items are noticed. The solidifying impact was higher in flour batter, where laccase activity is pervasive on arabinoxylan division, than in gluten batter, where laccases respond with gluten protein lattice with less effectiveness.

9.4.3 Wine Industry

Wine adjustment is one of the primary cycles requesting laccases in the food industry. Polyphenols in wine play a significant job in deciding the fragrance, shading, and taste of wine, which are carefully identified with the specific phenolic piece. In any case, similar mixtures are likewise the fundamental entertainers engaged with modernization measures causing turbidity, shading escalation, and fragrance and flavor modification. Laccase treatment pointed toward lessening of polyphenol amount, which shows a viable technique to safeguard wine quality, as the utilization of proteins can specifically eliminate target specific polyphenols without unfortunately modifying wine's organoleptic attributes. Laccases are appropriate with measure necessity, because of their solidness in acidic medium and reversible hindrance with sulfite.

9.4.4 Polymer Grafting

Laccase-interceded polymer adjustment covers unique perspectives in the paper industry. This capability has helped in biografting of phenolic acids for improving the strength properties of kraft paper produced using high kappa pulps. The main concept is to join phenolic acids to high-lignin content softwood kraft filaments intervened by a *Trametes villosa* laccase. The results show enhancements of hydrogen holding between strands and the making of phenoxy extremist crossconnects inside the sheets expanded paper strength.

9.4.5 Pharmaceutical and Cosmetic Industry

Laccases can catalyze a mix of various therapeutic products, for instance, antitoxins, antimicrobials, and antagonistic to illness drugs. The estimation of laccases for the amination of the new enemy of microbial has been represented. Various expected sweet-smelling alternatives of penicillins in addition to cephalosporins are common substrates of laccases. Novel cyclosporin analogs is procured by laccase-mediated oxidation. *Tricholoma giganteum, Lepiota ventrisospora, Lentinus edodes, Pleurotus cornucopiae, Coprinus comatus, Lentinus tigrinus, Hericium coralloides, Agaricus placomyces*, as well as *Abortiporus biennis* (laccase sources) have been uncovered as inhibitors against HIV-1 chat transcriptase activity. Laccase also has been represented to have the limit of combating aceruloplasminemia, restoring the iron homeostasis.

9.4.6 Paper and Pulp Industry

Paper is created from wood and comprises mostly cellulose; thus, it is important to eliminate the lignin from the wood mash. Presently, oxygen chlorine-based

delignification, oxygen delignification, or dyeing methods are utilized to treat the wood mash to make paper. Be that as it may, these strategies cause environmental concerns due to the substance squanders that are produced (e.g., H_2O_2 or chlorine subsidiaries) (Ausec et al. 2017; Yang et al. 2018). More harmless to the ecosystem elective is the utilization of compounds, which other than keep up better uprightness of cellulose because of its explicitness. Notwithstanding, there are still difficulties in regard to expenses of creation (S.R.C.). So a savvy, harmless to the ecosystem, and explicit cycle could be accomplished, utilizing extremophile chemicals, particularly laccase, which explicitly eliminates lignin, protecting the carb construction of wood mash.

9.5 Conclusion and Future Perspectives

Strength, half-time life, a measure of movement, and cost are the factors that hinder the utilization of compounds for biotechnological purposes. To search for business applications that are cost delivering, temp-subordinate reactant properties of laccases are generally significant. Extremophile living beings have been chosen to deliver proteins with vigorous attributes in regard to warm soundness, natural dissolvable obstruction, execution within the sight of metals or high salt conditions, and so forth; subsequently, these creatures address an important wellspring of compounds which, assuming appropriately separated and described, could connect the expense restricting the impact of denaturation of mesophile chemicals. Metagenomic prospection is additionally the most encouraging device for the confinement of extremophile laccases without the need of refined extremophile creatures, which exactly by their temperament could be an impedance (Batista-García et al. 2016). A significant bottleneck for getting mechanically savvy compounds is laccase articulation. Normally, yeasts have been a decision for laccase articulation since they are single-celled life forms that multiply in straightforward media and have a fast-multiplying time. Be that as it may, albeit some encouraging reports are emerging utilizing *Pichia pastoris* (Zerva et al. 2019). Best outcomes are gotten with filamentous parasites as host for the heterologous laccase qualities (Wikee et al. 2019). Even though laccases were initially found in plants, articulation in these living beings has ended up being troublesome (Legerská et al. 2016). Regardless of certain impediments, extremophile laccases still address the most ideal alternative to use for biotechnological applications.

Acknowledgments Dr. Surajbhan Sevda thanks NIT Warangal for the Research Seed grant (P1128) for a support of this work.

Conflicts of Interest There is no conflict of interest to declare.

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Pectinases: Production, Harvest, Recovery, and Potential Industrial Application

10

Riya Sahu and Surajbhan Sevda

Abstract

Pectinases are a group of enzymes that lyse pectin. Pectins are polysaccharides, and they are found abundantly in plant cell walls. The feature of pectinolytic activity is exploited by the industry. With the advances, the industry has developed a focus on various microbial sources for the efficient production of pectinolytic enzymes. The bioprocess principles are applied extensively for the production of pectinase enzymes for efficient commercial production and harvest. Many factors affect the yield of pectinases, which are overlooked, and the shortcomings are improved; this is done with the help of understanding and the research made on studying the biochemical properties of pectinases. The purification and characterization of pectinase enzymes have a key role in controlling the purity and standards. With the help of studies on the mechanism of action of pectinases, it is found to have wide applications, which range in the field of the brewery, juice making, jam making, retting, plant fiber making, paper making, and so on.

Keywords

Pectinases · Pectinolytic · Brewery · Retting · Pectin

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_10

Abbreviations

HG	Homogalacturonan
PE	Pectinesterase
PG	Polygalacturonases
PGL	Polygalacturonate lyase
PL	Pectin lyase
PMG	Polymethyl galacturonase
RGI	Rhamnogalacturonan I
RGII	Rhamnogalacturonan II

10.1 Introduction

Pectinases are enzymes of nice industrial importance because it contains a broad application ranging from paper-pulp industry, brewery, fruit juice industry, to retting of plant fibers, and so forth. Pectinases or pectinolytic enzymes are comprised of enzymes that have a feature to change the pectin present in plant parts. Pectinolytic enzymes are ample among microorganisms and higher plants (Jayani et al. 2005). They also have ecological significance as they decompose and recycle plant waste. The source of the pectinase enzyme can be microorganisms present in the site rich with pectin. In the current days food and agriculture industry waste, such as peels, can be reused more to go for a low price and economical production of pectinase enzyme with the assistance of microbes in an eco-friendly way. The study showed sensible production with low-priced substrates such as orange rind with the help of novel *Streptomyces fumigatiscleroticus* VIT-SP4 strain (Govindaraji and Vuppu 2020). As per market research trends, pectinase holds nice worth and secures a prominent position among commercially used enzymes and will touch about $41.4 billion in the forthcoming time (Garg et al. 2016; El Enshasy et al. 2018).

10.1.1 The Pectinolytic Enzymes

Figure 10.1 shows the classifications of pectinases on the basis of their interaction with various pectin substances. They are divided into groups as follows:

1. Protopectinases promote soluble highly polymerized.
2. Esterases enhance the reaction rate for de-esterification via methoxy ester elimination from pectin.
3. Depolymerases enhance the rate of reaction of cleavage (hydrolytic) of α-(1 → 4) glycosidic bonds in pectic substance's D-galacturonic acid.

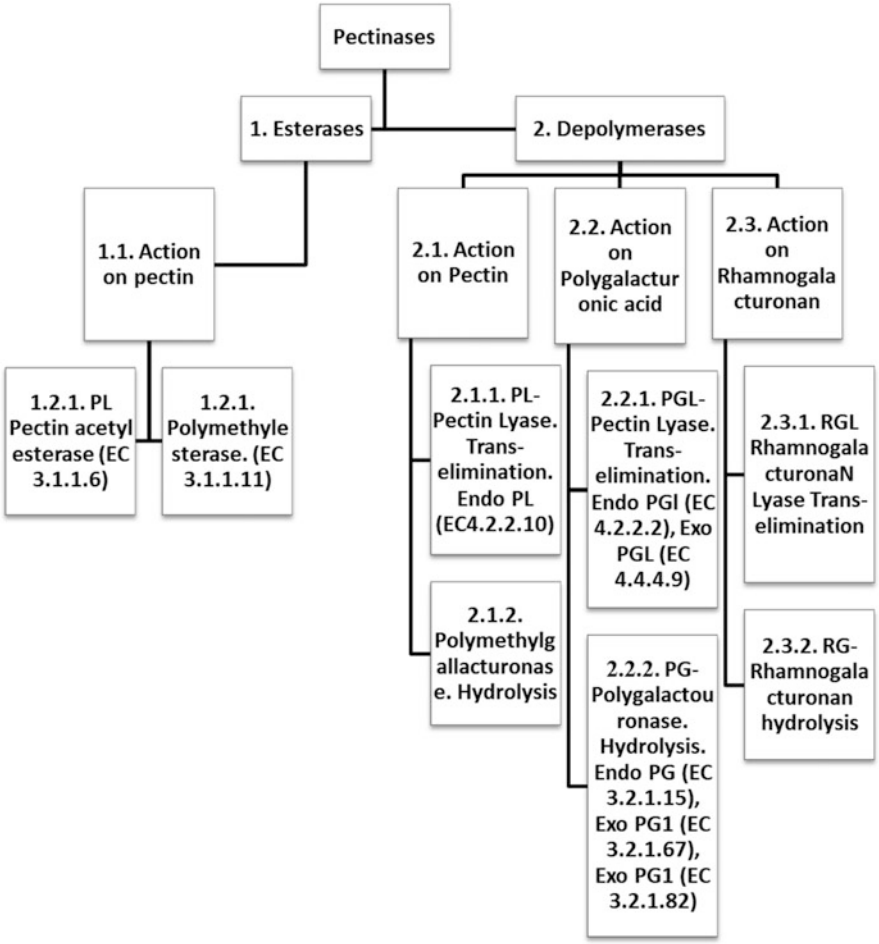

Fig. 10.1 Grouping of various pectinases based on their interaction with various pectin substances

10.1.2 Pectins

Pectins, abundantly found in plants, are polysaccharides and comprises combination of polygalacturonic acid and branched sugars. They are the substrates for pectinases. The location where it's found in plants includes the middle lamellae and plant cell wall (Agrios 2009). Pectins aid the physiological processes of plant tissue like cell differentiation—growth contributing to integrity and rigidity (Voragen et al. 2009). They're additionally involved in several functional aspects in plants, such as defense and damage mechanism, cross-linking cellulose, and hemicellulose fibers (Giacobbe et al. 2014). Cellulose polysaccharides participate in the regulation of ion transport, the porosity of membrane walls, and the permeability of the membrane for enzymes. They additionally decide the water-retaining capacity. The quantity and composition

of pectic molecules in vegetables and fruits and different plant produce strongly decide the most effective parameters of clean and processed edible products (Voragen et al. 2009). These are exploited in industries, and they can also be extracted from by-products of vegetables and fruits, such as pomace of apple peels of citrus fruit, because of their gelling, thickening, and stabilizing properties (Tapre and Jain 2014). Pectic substances, characterized as acidic complex colloidal polysaccharides, consisting backbone made up of α (1–4) linked galacturonic acid residues with side chains consisting-arabinose, galactose, xylose and, L-rhamnose. Upon modification of the backbone chain, they are further classified as (Bemiller 1986). Pectic substances as per the American Chemical Society are summarized in the following ways (Kertesz et al. 1944):

1. Protopectin: It gives pectinic acid or pectin upon hydrolysis. This can be a parent pectic substance and, upon restricted reaction (hydrolysis), yields pectin or pectinic acid. Protopectin is water insoluble, while its hydrolysis products are water soluble.
2. Pectic acids: Pectic substances are free of ester groups and methyl groups consisting of colloidal polygalacturonic acid. The salts of pectic acid are called pectates.
3. Pectinic acids: They are a combination of polygalacturonic acids, comprising of ester and methyl groups. The salts of pectic acid are called pectinates.
4. Pectins: They are a mixture of varying compositions, comprising of pectinic acid because of the major constituent.

10.1.3 Pectic Substance's Nature

High-molecular-weight pectic substances exist as pectins, which as per the polysaccharide are acidic and diverse (Doco et al. 1997). It is found in vegetables, grains, and fibers. In plants, it is found near the center lamella and cell wall and in regions containing an arrangement of cellulose and hemicellulose (Caffall and Mohnen 2009). The backbone of pectin has residues of D-galacturonic acid (Gummadi and Panda 2003), connected by α(1~4) linkage with a few rhamnose over the main chain and its side chain; it has galactose, arabinose, and xylose (Fig. 10.2). Galacturonic residues are present with a carboxyl group, adjusted at various regions with the methyl groups, which are esterified to offer grip to neighboring cells (Yadav et al. 2008). The esterification extent is variable in plants as some contain extra acetylated D-galacturonate. The gel structure of pectin is formed with the aid of divalent cations of calcium and magnesium, which connect carboxy groups that are not esterified. This plays a role in mechanical strengths and other physical properties and maintains structure and function attachment properties (Sun and van Nocker 2010), and they can be part of signaling-related defense and morphogenesis.

Pectin structure as atom model was proposed with backbone consisting of HG and RGII are RGI (Vincken et al. 2003). Components present in pectin structure (Fig. 10.2.) are as follows:

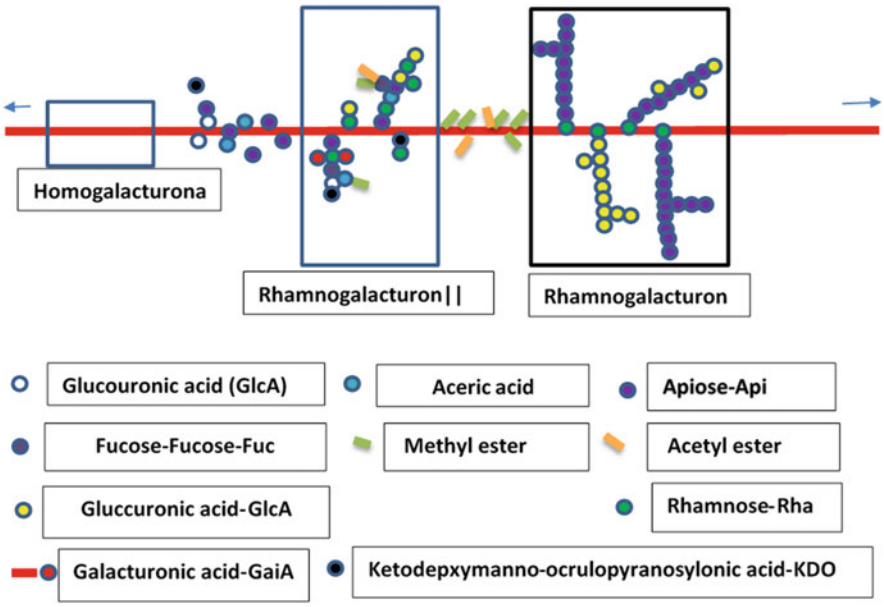

Fig. 10.2 Diagram showing structure of pectin

1. Homogalacturonan (HG): It's the plain or smooth portion of pectin containing. It is a polymer of esterified, methyl, or acetylated D-galacturonic acid. In the esterified form of polymer, it exists as polygalacturonic/pectic acid, pectin (methylated carboxyl group = 75%), or pectinic acid (methylated carboxyl group nearly 75%) (Jayani et al. 2005).
2. Rhamnogalacturonan I (RGI): It contains repetition of rhamnose and galacturonic acid (disaccharide) with side chains that could have sugars (arabinose, xylose, galactose) and galacturonic residue that can be acetylated.
3. Rhamnogalacturonan II (RGII): It resembles HG, differing in terms of side chains, which are complex and linked to galacturonic residues.

The first description for the structure of pectinase was described for pectinase Pel PelC/EchPL1; it was from *Erwinia chrysanthemi* family 1 (Yoder and Jurnak 1995). The structure of pectinase comprises eight complete beta-strand turns, forming three different beta sheets and a parallel beta helix. The entire pectinases have the same topography for the beta helix as found in the *Erwinia chrysanthemi* pectinase-Pel C. The core of the enzyme has hydrogen bonds among side chains—aromatic and aliphatic—which are repeated, and it aids stabilization (Herron et al. 2000).

10.2 Sources of Microbial Pectinases

Many microbes could produce pectinase enzymes; some of the pectinase-producing microbes are the following: *Aspergillus niger, Bacillus subtilis, Pseudomonas, Actinomycetes, Streptomycetes, Aspergillus versicolor, Rhizopus stolonifer, Penicillium jenseni, Penicillium citrinum, Xanthomonas, Aspergillus flavus, Mucor racemosus, Mucor hiemalis, Trichoderma viride, Fusarium oxysporum,* and *Erwinia* (Priya and Sashi 2014).

Successful production of various pectinases has been achieved with the help of microorganisms providing optimum conditions and suitable substrate, for example, *Aspergillus niger and Pectobacterium carotovorum* for pectin lyase, *Aureobasidium pullulans* for polygalacturonase with tomato-related agriwaste as substrate, *Penicillin notatum* for polygalacturonase with wheat bran as substrate, and *Aspergillus fumigatus R6* for pectinase and polygalacturonase with rice bran as substrate, respectively (Rebello et al. 2017).

Among fungi, *Aspergillus niger* is the source of pectinase; some of the strains yield enzyme, which has a good activity even at 100 °C and alkaline conditions such as MCAS2 pectinase (Khatri et al. 2015). Apart from this many other thermostable and pH-tolerant pectinases have been discovered:

- *Streptomyces* spp. QG-11-3 produce polygalacturonase; it shows good activity at 60 °C.
- *Thermotoga maritima* produces polygalacturonase; it shows optimal activity at 80 °C (Kluskens et al. 2005).
- *Bacillus* sp. BR1390 produces thermostable polymethylgacturonase, which shows 100% activity with 60 min of incubation at 60 °C (Rastegari and Karbalaei-Heidari 2014).

Thermotolerant and high-yield pectinases are combined to build recombinant high-yielding isolates with the application of production optimization strategies, metabolic engineering, metagenomics, and site-directed mutagenesis.

Recombinant DNA technology has also been applied in pectinase production and few important combinations of parent microbe and host for expression. The RDNA technology application in pectinase production *Pichia pastoris and Escherichia coli* is the most commonly used host for expression.

The RDNA technology applications and studies for pectinase production have shown *Pichia pastoris* as suitable host for expression for microbes: *Aspergillus aculeatus, Penicillium species CGMCC-1669,* and *Escherichia coli* as suitable host for expression for microbes: *Bacillus clausii, Bacillus halodurans,* and *Paenibacillus campinasensis BL-11*, respectively (Rebello et al. 2017).

10.3 Pectinase Production

Production approaches for pectin include solid-state fermentation as well as submerged fermentation. Different parameters, such as substrate choice, amount of inoculum, water amount, environment and condition of process conditions, duration of incubation, temperature, pH, sources for nitrogen and carbon, and presence or absence of inhibitors/activators, affect the biosynthesis of pectinase biosynthesis. Various substrates are chosen for pectinase production, which includes orange, shells of nut, rice bran, bagasse from sugarcane, wheat bran, and so on.

Some optimum aspects related to pectinase production with the help of various studies are:

- With studies, it is found that, among the diverse substrate options, wheat as a substrate was most ambient (Amin et al. 2017).
- Ammonium sulfate's role in pectinase production was found to be favorable for few *Aspergillus* species (Phutela et al. 2005).
- In the case of *Aspergillus fumigatus*, over the wheat grain substrate, it achieved good polygalacturonase production at optimum pH of 4.0 and 5.0. For pH 4.0, activity on pectin was found to be 1116 U g^{-1} while at 5 pH (Phutela et al. 2005).
- For solid-state fermentation in a pilot-scale bed reactor, agitation intermittently enhances the production of pectinase (Finkler et al. 2017).
- *Bacillus pumilus* dcsr1 shows more good production of thermostable alkaline pectinases in solid-state fermentation as compared to fermentation in submerged culture (Sharma and Satyanarayana 2012).
- It was found that, on the pulsed electric field exposure of pectinase, without affecting the structure, it increased thermal stability (Zhang et al. 2017).
- Pectinase on the treatment with gentle ultrasound showed better results for immobilization and enzymatic activity but decreased its reaction stability, reusability, and thermal stability because of underlying structural changes.

Based on various fermentation methods and suitable substrates, different bioreactors have been used to achieve pectinase production of the desired yield:

- Solid-state fermentation has been used for column tray yielding 2181 U/L with substrate as lemon peels, packed bed-recycled flow yielding volumetric productivity 0.98 U per ml per hour with grains substrate, pilot scale packed bed yielding 1840 U/kg of solid per hour with sugarcane bagasse plus wheat bran as substrate, rotating drum yielding 4 U g dm^{-1} with rice bran as substrate, and tray type bioreactor yielding 298 U/g of wheat bran substrate, respectively (Rebello et al. 2017).
- While with submerged fermentation method via stirred tank bioreactor, orange peel as substrate for exo-pectinase production gives yield of 670 U/L h in batch and 28.3 U/L h in pulsed fed batch, while for endo-pectinase production gives yield of 28.2 U/L h in batch and 24.89 U/L h in pulsed fed batch, respectively (Rebello et al. 2017).

10.4 Factors Affecting Pectinase Yield

Aspergillus niger is one of the great sources for the production of pectinase; the various factors affecting pectinases yield are as per below:

10.4.1 Time

In liquid culture production of pectin methylesterase by *Aspergillus niger* showed optimum growth with 6 days of culturing, and the best enzyme activity was observed on the fourth day. In the case of pectic enzyme production by molds, optimum production varies from 1 to 6 days based on different molds (Fawole and Odunfa 2003; Ghildyal et al. 1981).

10.4.2 Nitrogen and Carbon Source

For diverse molds, pectic substrates improved the yield of a pectic enzyme. Catabolite repression sensitivity of pectic enzyme was observed by high galacturonic acid and exogenous glucose (Aguilar and Huitron 1987). Pectic enzyme production was promoted when the carbon source in the medium used was from polygalacturonic acid and pectin.

The addition of different concentrations of glucose to the culture medium with pectin showed a different effect on enzyme. At higher concentration of glucose in the medium, the enzyme production was inhibited and on the other hand, were stimulated at a lower glucose concentration of 0.5% w/v. A negative effect was observed in the enzyme production with the presence of glycine and tryptophan, while a positive effect was observed in the presence of ammonium nitrate and ammonium sulfate (Fawole and Odunfa 2003).

10.4.3 Effect of Temperature

The ambient temperature for pectic enzyme production was found to be 40° in the case of *Aspergillus niger* reported by Fawole and Odunfa (2003), 30 °C in the case of *Aspergillus foetidus* (Hours et al. 1988), and 37 °C in the case of few another *Aspergillus* species (Aguilar and Huitron 1987). This study indicated that slight high temperature is favorable for enzyme activity. This was widely applied in the food industry.

10.4.4 pH

The acidic range pH was observed for most f the molds for the production of pectinase enzymes (Shin et al. 1993). For 5-day culture of *Aspergillus niger*

fermentation of pectin at 30 °C for 5 days, the optimum pH observed was 5 for best production (Fawole and Odunfa 2003).

10.4.5 Surface Culture Method

The action of pectin methylesterase was found to be more in the solid-state surface culture method as compared to the fermentation by the submerged method. Polygalacturonase activity was observed more in submerged than that in surface culture method. The surface culture method was found to support higher enzyme production (Fawole and Odunfa 2003).

10.4.6 Agitation

Agitation could support favorable for catabolite repression due to enhanced agitation of few potent substances which can cause it, inhibition like situation observed in *Aspergillus niger* pectic enzyme production (Fawole and Odunfa 2003).

10.5 Regulation of Pectinase Synthesis

The feature of microbial source for pectinase production is exploited commercially. *Aspergillus niger and Bacillus subtilis* are used commercially for the production of pectinase. Some examples include PectinexTM, Pectinex SP, etc., which are made and utilized by enterprises (Sutton and Peterson 2001).

For cost-cutting strategies, solid-state fermentation with substrates from agriwaste and fruit processing waste, such as orange peel, sugarcane bagasse, and banana peel, were reused and recycled more against the submerged fermentation method, which involves a costly medium for polygalacturonase. The broad application of pectinases, improvement of production technology, comprehension studies, and different recuperation strategies contributed toward the production of enzymes with the help of microorganisms (Patill and Dayanand 2006). For a higher industrial large-scale yield of pectinase, coordinated measures (upstream and downstream processing), execution of procedures like strain improvement, or any cutting-edge methods to enhance the yields of bacteria is required.

Numerous microorganisms produce enzymes, such as pectinases, cellulases, and amylases, that break the polymers into simpler sugars (Bruhlman et al. 1994). Along these lines, compound innovation is focused on pectinolytic microorganisms and, for the development of high-yielding strains, genetic modification for high-yielding strains of pectinolytic microorganisms production as they are cheaper, eco-friendly, and readily available (Suneetha and Khan 2011).

10.6 Purification and Characterization of Microbial Pectinases

10.6.1 Purification

Purification is important as purity is playing a role to study and characterize pectinases. Different combinations of purification methods are used, depending on the type of pectinase to be extracted and the source organism. Table 10.1. shows the various purification methods used for various pectinase generations.

10.6.2 Characterization

10.6.2.1 Physicochemical Characterization Pectinase Enzymes Are Characterized Based on Diverse Factors Related to Physical Features and Chemical Features

- *PMG—Polymethyl galacturonase:* The ambient pH is 4–5 for PMG production, and the ambient substrates are esterified pectin (95%) (Schnitzhofer et al. 2007).
- *PG—Polygalacturonases*.
  - *Exo-PG:* Their molecular weight lies between 30 and 80 kDa, and their ambient temperature, pI, and pH are found to be 30 to 50 °C, 3.8–7.6, and 2.5–6.0, respectively (Takao et al. 2001; Singh and AppuRao 1989).
  - *Endo-PG:* It has a molecular weight in the range of 30–50 kDa and pi between 4 and 6.
- *PL—Pectin lyase:* It is active in alkaline as well as acidic (pH 4–7) pH. It has molecular weight near to 30–40 kDa (Hayashi et al. 1997; Soriano et al. 2005). It has pi near to 3.5 (Moharib et al. 2000; Sakiyama et al. 2001).
  - *PGL—Polygalacturonate lyase:* It has a molecular weight of 30–50 kDa. In *Bacteroides* and *Pseudoalteromonas*, it has a higher molecular weight near to 75 kDa (McCarthy et al. 1985; Truong et al. 2001). The ambient pH for PGL is between 8 and 11, while for *Bacillus licheniformis*, and it is 11 and 6, respectively.
- *PE—Pectin methylesterase or pectinesterase:* It has a molecular weight of 30–50 kDa (Hadj et al. 2002; Christensen et al. 2002). The ambient pH of PE is between 4 and 7, in general, for *Erwinia* it is near to alkaline pH. Their ambient temperature lies between 40 and 60 °C. They have pH between 4 and 8.

10.6.2.2 Molecular Characterizations of Pectinase

For two strains of Aspergillus niger 4M-147 and N400 originated PNLA-pectin lyase A, the crystal structure comprises the parallel beta sheet. Their features related to structure resemble PL-like amino acid stacking, asparagine pattern. They have a sequence identity of 17% upon pairwise alignment. Substrate binding sites in them have negative electric potential containing mostly aromatic amino acids. The two strains differ in the region of 182–187 residue with diverse loop confirmations because of the diverse pH of crystallization (Mayans et al. 1997). Tailoring of degenerate primer for PNL gene PCR amplification can be exploited for recombinant

Table 10.1 Different purification method used for various pectinases

Source microorganism	Enzyme	Purification method	References
Fungal system			
Aspergillus Niger	Exo-PG	DEAE elution (sodium acetate buffer) Activity—increase by 209-fold (presence of Mg^{2+}) Recover—8.6%	Mill (1966)
Trichoderma reesei	Exo-PG, endo-PG, and pectinesterase	Sephadex chromatography	MarkoviÄ et al. (1985)
Rhizopus stolonifer	Polygalacturonase	Ethanol precipitation Ion exchange chromatography (CM-Sepharose 6B) Gel filtration (Sephadex G-100) Activity—tenfold	Manachini et al. (1987)
Aureobasidium pullulans LV10	PG and PL (pectin lyase)	CM-Sepharose 6B Column chromatography (DEAE-cellulose column) Gel filtration (Sephadex G-100)	Manachini et al. (1988)
Bacterial system			
Amycolata species	Pectate lyase (PGL)	Cation and anion exchange columns Hydrophobic interaction chromatography Activity—fourfold increase Recovery—37%	Manachini et al. (1988)
Clostridium aectobutylicum ID 91-36	Pectinases	Cation exchange chromatography (Sepharose column, NaCl elution)	Brühlmann (1995)
Bacillus macerans	Endopectate lyase	Ammonium sulfate precipitation DEAE-Sephadex A-50 chromatography and CM-Cellulofine	Miyazaki (1991)
Erwinia carotovora	Endopectate lyase I–IV	CM Sepharose CL 6B chromatograph Sephadex S-200 gel filtration Isoelectric focusing	Tanabe et al. (1984)
Aspergillus awamori IFO 4033	Pectinases	Cation exchange Size-exclusion chromatographic columns	Nagai et al. (2000)
Erwinia caratovora FERM P-7576	Endopectate lyase	Selective co-sedimentation Cell-free broth precipitation Gel chromatography separation Activity—710 U/m g	Fukuoka et al. (1990)

Table 10.2 Bioinformatics approaches to study pectinases to understand molecular properties

Function	Bioinformatics approach used	Source
Multiple sequence alignment	Seaview and Clustalw	Lassmann and Sonnhammer (2006)
Dendrogram construction	Mega 3.1 (minimum evolution, neighbor joining, and UPGMA methods)	Kumar et al. (2004), Salemi and Vandamme (2003)
Domain search	Pfam site	Cao (2012)
Domain analysis to find conserved motifs	MEME	Yadav et al. (2009)
Domain study and similarity score prediction	Interproscan	Yadav et al. (2009)

expression, and this can be done by multiple sequence alignment of various organism PNL protein sequences with the help of programs such as Seaview and Clustalw.

The data on sequences of all pectinases are available in NCBI and GenBank database. All the sequences of PNL, PL, PG, and PE of different source organisms are available in GenBank from NCBI. A total of 494, 717, 937, and 172 protein sequences of PNL, PL, PG, and PE, respectively, represent major groups of pectinases (Table 10.2).

10.7 Biochemical Properties of Pectinases

Esterases and depolymerases are subcategories of pectin. Esterases catabolize ester to water and alcohol, while depolymerase performs hydrolysis and trans-elimination reactions.

10.7.1 PMG: Polymethylgalacturonase

The activity of PMG can be analyzed by reduction of substrate viscosity or determine reducing sugars which are formed upon hydrolysis of glycosidic bonds by PMG. Microbes that yield good PMG majorly include *Aspergillus* species, *Botrytis*, *Penicillium* species, and sclerotium species.

10.7.2 PG: Polygalacturonases

The action of PG involves hydrolysis of glycosidic linkage found in polygalacturonase via endo- or exo-mechanism. It can be estimated similarly as measured for polymethylgalacturonase, but for exo-PG viscosity, change method is not efficient. The microbial assay cup-plate method is also helpful to analyze enzyme

action (Truong et al. 2001). The major source for endo PG includes organisms, microbes, and yeast.

10.7.3 PL: Pectin Lyases

The action of endo-PL includes catabolism of pectic substances in an arbitrary style which produces 4:5 unsaturated oligo methyl glucuronate is PL isolated from *Aspergillus niger* performs trans-elimination activity on gelatin and hence its de-polymerization (Van Alebeek et al. 2002). The activity of PL can be analyzed by the reduction in substrate viscosity. Generally, they do not require Ca^{2+} ions presence for their action except for Fusarium pectin lyase, which requires methylesterification of the galacturonic acid (Hayashi et al. 1997; Soriano et al. 2005).

10.7.4 PGL: Polygalacturonate Lyase

Exo-polygalacturonate lyase and endo-polygalacturonate lyase degrade and break pectate via trans-elimination reaction, which produces 4,5 unsaturated oligo galacturonates. PGLs are discovered uniquely in microorganisms, and they require Ca^{2+} for movement. Ambient pH for PGL activity is between 6 and 10, while pectinases have lower ambient pH in which they are active (Singh et al. 1999; Dixit et al. 2004; Truong et al. 2001).

10.7.5 PE: Pectinesterase or Gelatin Methylesterase

It does not act on pectates and only acts on pectins that are esterified action of PE involves hydrolysis of galacturonan pectin backbone, in which methoxy group is removed from 6-carboxyl gathering, which produces hydrogen ion, methanol, and pectic acid. Pectinesterases are fundamentally delivered for plants such as banana and orange and by bacteria and fungi (Sharma et al. 2012).

10.8 Mechanism of Action of the Pectinases

Grouping of pectinase enzymes and their mechanism is decided under three divisions based on (a) for substrates like pectin or pectic acid or oligo-D-galacturonate, if cleavage is within then it will be endo and the result will depolymerization, in the case of endwise it will be exo and will result in saccharification. and if the pectinases perform hydrolysis or trans elimination (Blanco et al. 1999; Sakai 1992; Whitaker 1990). (b) Based on their mechanism as mentioned above, they can be divided as in Fig. 10.3.

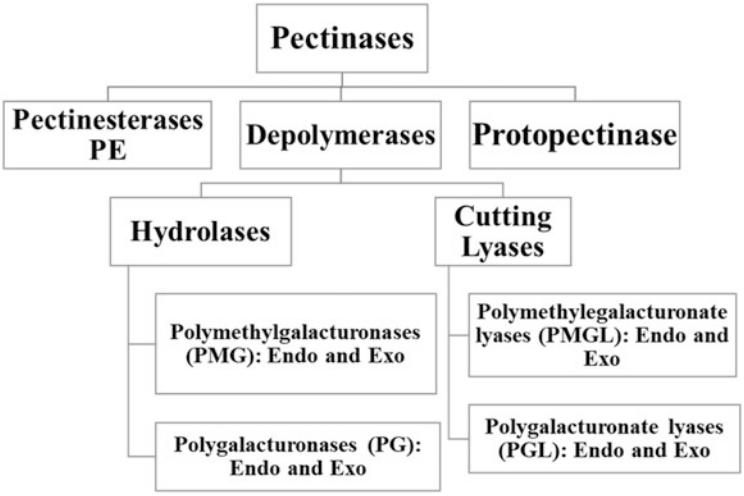

Fig. 10.3 Pectinases and its types based on mechanism

10.8.1 Mechanism

10.8.1.1 Pectinesterases (PE)

They are otherwise called pectin methyl hydrolase, aid the catalysis of de-esterification of methoxyl part in pectin galacturonate portions, and form PA-pectic acid.

10.8.1.2 Depolymerases

Pectic substances have D-galacturonic acidic regions; these are hydrolyzed by depolymerases (α(1, 4)-glycosidic bonds) (Rexová-Benková and Marković 1976). Depolymerases hydrolyzing glycosidic linkages. They include polymethylgalacturonases (PMG) and have α(1,4)-glycosidic bond hydrolysis.

10.8.1.3 Cutting Lyases

Formation of galacturonide comprising of C4–C5 unsaturated bond via trans-elimination reaction cleaves α(1,4)-glycosidic linkages, near the nonreducing.

10.8.1.4 Protopectinase

They form polymerized soluble pectin by solubilizing protopectin (Tapre and Jain 2014).

Pectinases are encoded by an enormous family which is multigenic, which has wide pathways for expression with a specialized function (Coutinho et al. 2003). These families are the results of the duplication of a gene that is connected tandemly (Carroll et al. 2005).

Expression of pectin methylesterase and pectin lyase is found to be induced by D-galacturonic acid, polygalacturonic acid, and pectins. Repressors associated with

these are glucose and Cre A—a carbon catabolite repressor protein (Maldonado and Saad 1998). The presence of conserved sequence has been concluded from promotion deletion studies and researches: 5′-TYATTGGTGGAA-3′ on pectinases, which engage in the expression of a gene by initiation and activation (Visser et al. 2004).

10.8.2 Active Site in Pectinase

Pectinase catalysis starts with the binding of the substrate on the glycosidic bond-susceptible site through various hydrogen bonds and creates appropriate changes to accommodate at the active site. His223 passes a proton to glycosidic bond, which leads to glycosidic linkage cleaving, delivering the primitive product and another covalent bond form among substrate with the active site of asparagine Asp 201 nucleophile. While other Asp202 residue locates a molecule of water for nucleophilic attack for second product formation and reestablish the active site of the enzyme (Palanivelu 2006). Hence, ionization of His and Asp is influenced by pH conditions (Kohli and Gupta 2015).

10.9 Biotechnological Potentials of Pectinases

Unraveling of gene information along with the recombinant DNA technology leads to the making of strains that produce an enzyme in the bioreactor with higher yield and less cost (Kumar and Verma 2020a, b). Pectinases have a wide and interdisciplinary potential in biotechnology. Some of the enumerated are as follows:

- **Acid and alkaline pectinases: Acidic pectinases** are used widely in the beverage and brewery industry. Examples: Fruit juice making and winemaking(Kohli and Gupta 2015).
- **Plant virus purification:** They have also been employed in the purification of plant viruses. They are yet to be commercialized (Salazar and Jayasinghe 1999).
- **Disease control in plants:** Pectin lyase obtained from *Bacillus clausii* is found to impart resistance against plant disease *Cucumis sativus* seedlings, hence also playing a role as a biocontrol agent, and this also reduces chemical usage (Li et al. 2012).
- **Safer fruit juices:** Pectinases can break down pectins, which are esterified more. For example, fruits broke down into small molecules by the mechanism of beta elimination in which no methanol is produced. Methanol may be toxic and reason of health hazards (Whitaker 1990).
- **Degumming and retting applications:** Alkaline pectinases are used in the textile industry for the retting purpose of plant fibers such as jute, hemp, flax, ramie, and so on.
- **Stability and chromaticity enhancement in the brewery industry:** In the brewery industry used in the red winemaking process, which gives the additional

benefit of improved chromaticity and stability (Revilla and Gonzalez-San Jose 2003).

- **Oil extraction:** Oils from fruits such as orange and lemon can be obtained. They adversely affect the emulsification of pectin.
- **Makes commercial and industrial process efficient (filteration time and pressing efficiency):** Pectin solves and balances fruit juice viscosity and turbidity. Pectinases along with amylases are applied for juice clarification. The major benefit of this is it **reduces the filtration time** by half (Blanco et al. 1999). Pressing efficiency for fruits can also be enhanced by the combination of pectinases with arabinases, xylanases, and cellulases (Gailing et al. 2000). Acidic pectinases are widely used in the fruit industry as fruit juices have acidic pH (Jayani et al. 2005).
- **Freestone peach infusions:** With pectin methylesterase and Ca^{2+} keeps fruits firmer. This might be used for pickle preparing, where undesirable softening might happen while storage (Baker and Wicker 1996).
- **Bioscouring:** Pectins could also be utilized for the removal of noncellulosic components such as pectin, from the cellulosic matrix of the primary cell wall; the process is also known as bioscouring. This is used in cotton fibers and textile processing.
- **Fermentation of coffee and tea:** Alkaline pectinases improve the fermentation process and product by degrading undesirable pectin. In the case of coffee, it degrades the mucilage layer over coffee beans (Carr 1985).
- **Prebiotic component for animal feed:** Used in the production of animal prebiotic food parts (Sabajanes et al. 2012).
- **Pretreatment of pectin containing wastewater:** Removes pectinaceous material and aids faster degradation.
- **Kraft pulp biobleaching:** Used in paper and pulp industry. Pectin presence weakens dewatering while forming paper sheets and due to which yellowish appearance of paper is there (Viikari et al. 2001).
- **Recycling:** Food processing industry, agricultural wastes, de-inking of paper, lower BOD COD for waste with degraded pectin (Bajpai and Bajpai 1998).

10.10 Conclusion and Future Prospects

Pectinase's role related to immunology and plant microorganism virulence factors has got significant consideration will continue to be the thrust for future. Further improvement of such examinations will give a more extensive future research pectinase.

Additionally, not many data are accessible on the movement of these proteins post-synthesis and localization (Reeves et al. 1994; Russel 1998). More endeavors are required to get the idea of the transport of these proteins from the synthesis site and transport inhibitor estimation. These examinations will be useful for anticipation of numerous pathogen-related diseases. Studies have indicated that microbial pectinase can be used in the process of concrete crack healing and can also be

used for drug delivery applications in the future. Attention is required on cell biology for pectinase motioning in plants and microorganisms. Instruments, techniques and innovation, and knowledge in experiments could offer loreals in research over pectinase.

Acknowledgments Dr. Surajbhan Sevda thanks NIT Warangal for the Research Seed grant (P1128) for a support of this work.

Conflict of Interest The authors declare there is no conflict of interest to declare.

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Production of Malt-Based Beverages

11

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Abstract

A malt beverage is a fermented drink that is generally made from cereal grain such as barley that has been malted that involves germinating the grain in water followed by drying. This process converts the starch of the cereals to fermentable sugar, which is fermented by yeast to produce alcohol. Malting is an important process which assists the breakdown of cell wall of the grains and also contributes to the tastes and aroma of the drinks produced from it. Depending upon the alcohol content, the alcoholic beverages can be categorized as beer, wine, and distilled liquor. Beer is an example of such product, which is the most common malt-based alcoholic beverage in the world. It is an alcoholic beverage with low content of ethanol usually 1–10%, which is produced by the fermentation of malted barley along with hops (*Humulus lupulus*). Normally fermentation is achieved by yeasts *Saccharomyces cerevisiae*. The *Saccharomyces cerevisiae* is used to produce ale via warm fermentation, while the *Saccharomyces pastorianus* is used to produce lager at low temperature. Hops are used to give bitter taste to beer and also act as a preservative. Wine is a fruit-based fermented product made from grapes and wide variety of other fruits. Most wines are made from grapes belonging to the genus *Vitis vinifera,* which are rich in anthocyanins, hence giving color to these grapes. The unique composition of fruit juice gives wine its characteristic taste and aroma. Distilled liquors are produced by distillation of fermented drinks made of grains, fruits, or vegetables and contain higher percentage of alcohol compared to beer and wine. Examples of distilled liquors include whisky made from distillation of fermented grain mash; other examples include brandy derived from fermentation of wines or any fruit. Though alcohol

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_11

production has been for thousands of years, there's a lot more to alcohol production than just conversion of sugar to alcohol, it involves a complex process that have to take in to account the flavor, aroma, and even color of the finished product.

Keywords

Alcohol · Beer · Wine · Distilled alcohol · Fermentation · *Saccharomyces cerevisiae*

Abbreviations

ABV Alcohol by volume
EMP Embden-Meyerhof-Parnas
MLF Malolactic fermentation

11.1 Introduction

A malt beverage is a fermented drink that is generally made from cereal grain, such as barley, that has been malted that involves germinating the grain in water followed by drying. This process converts the starch of the cereals to fermentable sugar, which is fermented by yeast to produce alcohol. Malting is an important process that assists the breakdown of the cell wall of the grains and also contributes to the tastes and aroma of the drinks produced from it. Depending upon the alcohol content, the alcoholic beverages can be categorized as beer, wine, and distilled liquor. Beer is an example of such a product, which is the most common malt-based alcoholic beverage in the world. In earlier times, brewing was done on small scale by agrarian people (people connected with farming and agriculture) for domestic or low-scale commercial purposes, and this supplied beverage which served as a staple component in their diet. But the present globalization has changed the scenario, with growing big industries and hence large-scale production of alcoholic beverages. Thus, these changes bring about an alteration in the economy, the scale of production, techniques, and the nature of the product. There are two schools of thought regarding the origination of alcohol which finds deep root in agriculture. One school of believers (the larger group) has an opinion that agriculture started prior to alcohol production, whereas the other (smaller) group of people believe that agriculture developed because of the desire of people to drink alcohol. So, the concept finds similarity with the debate of which came first, the hen or the egg? (Katz and Voigt 1986). This makes us look back at factual artifacts of history. Historians have found that the ancient Egyptians practiced brewing, and they used to make beer from sorghum, a home-grown cereal that is still been practiced by African tribal people. Mesopotamians practiced brewing at around 2800 BC, and it has been found to be

the oldest handwritten proof of beer consumption in history to date (Hornsey 2004). It was from Germany that the use of hops in brewing spread throughout Europe from the tenth century.

11.2 Production of Beer

Beer is a popular fermented malt beverage found in all countries and almost all cultures. The process of beer production is known as brewing (Jay 1982). It is made through the conversion of sugar present in carbohydrate-rich cereals, which are facilitated by microorganisms and most commonly the yeast. Due to industrial standardization for quality control, industries have restricted beer production only with certain strains of yeast and no bacteria (Tyakht et al. 2021). But with the global need for large production, alternate methods are coming into practice in recent times. The end product of the fermentation is alcohol and the desired one is ethanol (Adams and Moss 2005). Wort is a sugar-rich liquid, containing trace elements, nitrogenous and sulfur compounds, that goes under controlled fermentation (Aroh 2018) Fermentation can be described by the following chemical reaction:

$$C_6H_{12}O_6 + 2PO_4^{3-} + 2ADP \rightarrow 2C_2H_5OH + 2CO_2 + 2ATP \quad (11.1)$$

11.2.1 Different Steps in the Production of Beer

The brewery process involves the alteration of malt into beer. For this, malt has to undergo the following processes:

11.2.1.1 Malting

Malting is a process in which the raw carbohydrate substrate is mixed with water, which results in the production of a liquid medium suitable for fermentation by the microorganism. This liquid mixture is known as "wort." Malting is an essential step in beer production, because the maximum carbohydrate used for the brewing of grains is starch and the yeasts which are used in beer fermentation are incapable of producing amylases. Hence, malt and, in certain cases, exogenous amylase source is supplied for the yeast (Jay 1982). The process of malting starts with cleaning grains, such as barley, which is then soaked in water for about 1–2 days. The grains are then spread on the floor to germinate. In this process of germination, the endosperm is attacked by the hydrolytic enzymes (α- and β-amylases), which are produced in the surrounding layer of endosperm, which is known as the aleurone (protein) layer. The function of α-amylase is to liquefy starch and β amylase increases sugar formation. Due to the hydrolytic activity, nutrients present in the endosperm are released properly for the growth of the barley plant. Sometimes, in order to facilitate this process, gibberellins, a plant growth hormone, are also added from an external source (Adams and Moss 2005) (Fig. 11.1).

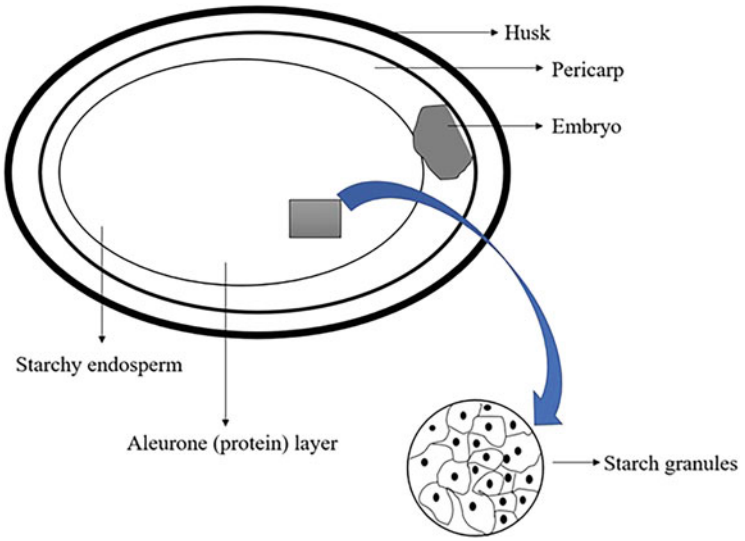

Fig. 11.1 Diagrammatic representation of barley grain

11.2.1.2 Milling and Mashing

In order to increase the surface area for effective contact between water (brewing liquor) and malt, milling (or grinding) of malt is an important step before mashing. Milling can be performed in two different ways: dry milling, which is performed by the majority of breweries, and wet milling, which was suggested by Lenz (1967), many years ago. During a comparative study (Szwajgier 2011), wet milling was found to improve the rate of extraction of sugars for fermentation from the filtration bed to the wort, which reduces the time for lautering. In addition to that, protein extraction also increased during wet milling, which has to be checked for the prevention of haze formation. The wet method also had an advantage over dry milling in reducing the phenolic compounds released during mashing, hence improving the beer colloidal stability (Pires and Brányik 2015) The central part of brewing is mashing, determining the nature of wort, which in turn determines the nutrient availability for yeasts to produce beer.

Mashing methods are of three types: (a) decoction methods, in which portion of mash from mash turn is transferred into mash kettle and boiled; (b) infusion methods, in which temperature is raised slowly without boiling the mash; and (c) double mash method, in which starchy adjuncts are put into the malt (Okafor 2007) (Fig. 11.2).

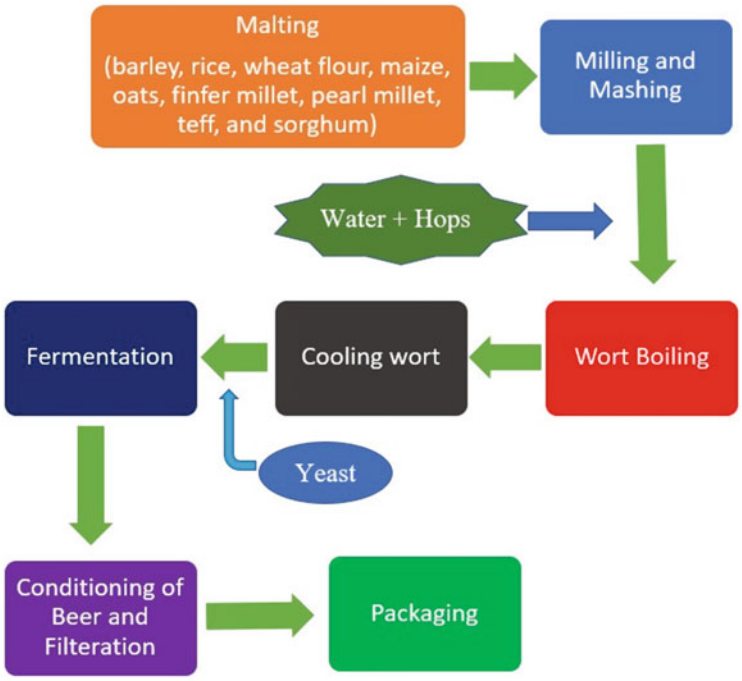

Fig. 11.2 Schematic representation of beer production

11.2.1.3 Boiling

After the transfer of wort into the kettle, the boiling process is done for 90–-120 minutes, which results in the evaporation of 5–15% of the total volume. (Miedaner 1986). At the beginning of this stage, hops are added into the kettle for flavor aroma and characteristic bitterness of the beer. Hops are generated from the cones (or strobili) of the female *Hamulus lupulus* plant. These plants are grown especially for the production of beer. The resinous yellow-colored powder, and essential oils are secreted by the lupulin glands of the female *Hamulus lupulus* plant. Hops give the beer a bitter taste and a typical aroma, maintain a balanced and stable microbial load in beer, enhance the foam lacing, and also help in the stabilization of foam in beer (Almaguer et al. 2014). With the help of a whirlpool effect, remaining hops, enzymes, and proteins are circulated throughout the media, which get coagulated and settle down, which can be removed from the bottom. This coagulated sludge is removed from the liquid mixture and is known as trub. Removing of trub from the kettle base gives surety that the bitter wort is clear and good to pass through the next stage of cooling (Adams and Moss 2005) (Fig. 11.2.) (Table 11.1).

Table 11.1 Aims of wort boiling

Aims of wort boiling	*Aims of wort boiling*
	To evaporate some amount of water
	To ensure the sterility of wort
	Inactivation of enzymes
	To make the wort concentrated
	Evaporating unwanted flavoring substances like DMS (dimethyl sulfide)
	Conversion of partially soluble α-acids (humulone and cohumulone) present in hop resin into a more wort-soluble form, i.e., isohumulones
	Precipitation of residual coagulated proteins
	Changing the color of wort to some extent

11.2.1.4 Cooling

Wort Cooling should be done as soon as possible to the minimization of the risk of infection. The aimed temperature should be between 15 and 25 °C in the case of top fermentation and between 5 and 10 °C in the case of bottom fermentation (Wunderlich and Back 2009).

11.2.1.5 Fermentation

After cooling and aeration, the wort is quickly mixed with suspended yeast cells in order to avoid contamination. This process of mixing is known as "pitching." Yeasts start to assimilate fermentable sugars, minerals, amino acids, and other nutrients soon after pitching. After pitching, yeast cells start to produce a wide range of metabolites, such as CO_2, ethanol, esters, and higher alcohol (Landaud et al. 2001; Pires and Brányik 2015). Conversion of fermentable sugar to ethanol by yeast takes place by EMP pathway (Adams and Moss 2005). Lager beers (beer conditioned at low temperature) are produced by *Saccharomyces uvarum* (*carlsbergensis*), which are bottom-fermenting yeasts, whereas ales (beer fermented or brewed using warm fermenting methods) are produced using *Saccharomyces cerevisiae*, which are top-fermenting yeasts (Eskin 1990).

Ale Warm fermented beers or the beers that are fermented by top-fermenting yeasts (yeasts that ferment at the top portion of vat) are known as "ale." They ferment quickly within 2–7 days and therefore can be consumed after few weeks of fermenting. Although they can be matured and conditioned for many months (Oliver and Colicchio 2012). The common top-fermenting yeast for brewing, i.e., *Saccharomyces cerevisiae*, is used in the production of ale, as they grow at a higher temperature range of 15–20 °C and sometimes at around 24 °C as well (Piggot and Lea 2003). Top fermenters work vigorously and form CO_2 at a rapid rate, which results in forming a thick mat of foam that can be taken out and used for starting the next batch of fermentation (Fig. 11.3.).

Lager Cool fermented beers or the beers that are fermented by bottom-fermenting yeasts (yeasts that ferment at the bottom portion of vat) are known as "lager."

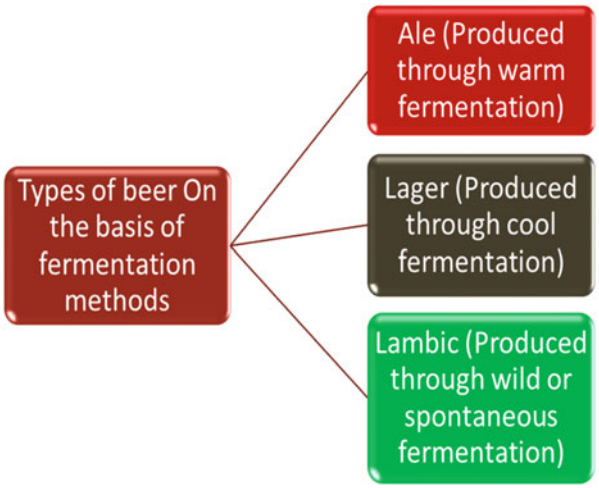

Fig. 11.3 Types of beer based on fermentation temperatures

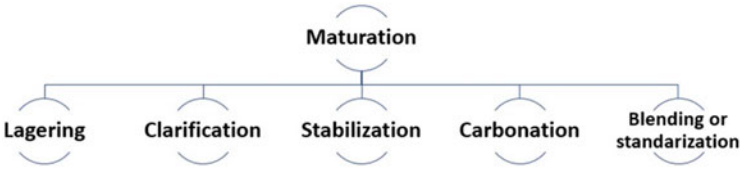

Fig. 11.4 Five parts of maturation or aging or conditioning

Bottom-fermenting yeast, such as *Saccharomyces pastorianus* and *Saccharomyces uvarum* (*carlsbergensis*), ferment efficiently at lower temperatures (5–10 °C). They do not work vigorously like the top fermenters, and CO_2 is formed at a much slower rate (Oliver and Colicchio 2012).

Spontaneous Fermentation or Wild Fermentation This type of fermentation done in a traditional manner using wild microbes especially *Brettanomyces*, which is a kind of yeast that is known for imparting beer a funky flavor. Lambic beer is an example of such a kind of fermentation (Verachtert and Iserentant 1995).

11.2.1.6 Conditioning or Maturation or Aging

After the use of all the fermentable sugar in the medium, the process of fermentation slows down. The yeasts then start to settle and move towards the bottom of the tank. Conditioning of beer takes place after the primary fermentation is finished. This process reduces the unwanted aromas and flavors from the immature or green beer after initial fermentation and converts it into a mature product. The end product after this process is expected to be a clear product (Pires and Brányik 2015). There are five parts into which maturation or aging or conditioning can be divided (Fig. 11.4).

Lagering, also known as cold aging, is a method that brings about maturation in the flavor of beer.

Clarification is done to clear out residual suspension particles and yeasts generated at the time of cold storage.

Stabilization is necessary to avoid changes in packaged beer. The possible changes that may occur due to inadequate stabilization include oxidized flavor changes, molecular and nonbiological complex formation resulting in hazy appearance, undesirable flavors due to contamination, and unwanted microbial growth, which also produces haze.

Carbonation is a process in which the concentration of CO_2 in beer is adjusted to a definite concentration. Traditionally, the adjustment was done by secondary cold fermentation, which was later replaced by injection of CO_2 directly from an external source.

Blending or stabilization is done for making batches of beer uniform (Fig. 11.4.)

11.2.1.7 Filtration

For microbiological stability, clarity of beer, removal of yeast, aroma, and extra flavoring compounds, filtration of the final product is done (Priest and Stewart 2006).

11.2.1.8 Flash Pasteurization

To ensure the shelf life of beer for months, pasteurization is done. There are several ways of pasteurization, of which the commonly used these days is flash pasteurization. In this process, beer is pasteurized at 75 °C for some time. But note should be taken not to overdo it, as it may degrade the flavor of the beer. Toward the end of the nineteenth century, tunnel pasteurization was developed, in which the exposure time was about 20 min at a constant temperature of 60 °C. Control of temperature and exposure time are the two very important factors in pasteurization (Fricker 1984; Eskin 1990).

11.2.1.9 Filling and Packaging

Around the 1870s, a machine for bottle blowing was developed, which could produce low-cost bottles. These days, dark, translucent bottles are used in the bottling of beer to maintain the flavor. Filling and packaging need to be contamination-free, safe to drink, and also look presentable and attractive to consumers without losing the quality (Table 11.2.). It is the expensive part of brewing and requires a sufficient number of laborers (Lowe and Elkin 1986; Eskin 1990).

Table 11.2 Points to be taken care of during filling

Points to be noted during filling of beer	
Bottling under pressure	If the partial pressure of dissolved CO_2 falls down, then CO_2 is released. Hence, packaging should be done at a particular pressure
Avoiding air contact	Decrease in flavor and colloidal stability of beer results from lower concentration of O_2 (about 0.01 mg/L)
Exhaustive cleaning	Proper cleaning regime has to be followed to avoid contamination of spoilage microorganisms

11.3 Wine Production

Wine is a fruit-based alcoholic beverage produced by the fermentation of sugar-rich sources like fruits especially grapes or grape juice. Fruit juices are considered to be the ideal substrate for alcoholic fermentation, because of the sugar content and nutrients along with ingredients like pigments, antioxidants, vitamins, and polyphenols (Kaur et al. 2019). The unique composition of fruit juices is mainly responsible for the taste and the aroma that develops during wine production (Piggot and Lea 2003). The production of wine by alcoholic fermentation worldwide is carried out by the yeast species, which is commonly found on grapes and vineyards and is therefore naturally occurring in wines. *Saccharomyces cerevisiae*, also known as "wine yeast," is the dominant microorganism found to be involved in the alcoholic fermentation of grapes; however, only 50 CFU/g of *Saccharomyces* was found to be present on grapes. The genetic diversity of *S. cerevisiae* strains results from the combination of their natural genetic diversity also referred to as non-domesticated yeasts and other domesticated yeasts. The evolution of the species is also influenced by stressful environmental conditions, and human interference has led to the development of industrially important strains with adaptive properties (Mortimor 2000; Brice et al. 2018). Many other species of yeasts, which are termed as non-*Saccharomyces* yeasts, which include *Ascomycota* and *Basidiomycota* phyla, are present in grape juice that play a role in the first stages of fermentation and also in the organoleptic property of wine (Fleet 2008; Kacaniova et al. 2020). Some of the non-*Saccharomyces*, also known as "wild yeast," found are *Brettanomyces, Candida, Debaryomyces, Kloeckera apiculata, Metschnikowia, Candida, Cryptococcus, Pichia, Rhodotorula, Zygosaccharomyces*, or *Kluyveromyces* (Boulton et al. 1995; Khan et al. 2000; Kantor et al. 2015). The initial phases of spontaneous and inoculated fermentation are usually carried out by non-*Saccharomyces* species, and in the successive stages, different *S. cerevisiae* strains are established. Several factors impacting the variability of grape microflora are the climatic conditions, localization, cultivar, use of pesticides, stage of grape ripening, the health of grapes, and harvesting procedures (Epifanio et al. 1999; Brilli et al. 2015; Padilla et al. 2016). After several years of research in the past recent years, it is considered that the non-*Saccharomyces* are important organisms for the wine fermentation process. Moreover, *S. cerevisiae* alone is unable to complete wine fermentation; therefore, they are mostly used as mixed starters along with the non-*Saccharomyces* species (Vilela 2020). Differences were found in the metabolic profile of compounds in wines made from the starter mixed culture and monoculture fermentation. Additionally, the volatiles cannot be predicted from the knowledge about yeast strains in mixed fermentation (Howell et al. 2005). *S. cerevisiae* metabolizes sugar by fermenting it into ethanol and carbon dioxide as the primary fermentation metabolites (Walker and Stewart 2016). Yeasts also produce a plethora of other secondary metabolites, like volatile fatty acids, higher alcohols, esters, aldehydes, volatile phenols, and sulfur compounds, which are responsible for imparting characteristic flavor and aroma to the final wine (Strauss et al. 2001; Romano et al. 2003; Viana et al. 2008; Renault et al. 2009; Whitener et al. 2015; Englezos et al. 2015). It

has been found that the non-*Saccharomyces* species produce enzymes during the fermentation process which contribute to the complexity and organoleptic characteristics of wine. Primary aromatic compounds are formed during fruit ripening and are present either in free or bound form and belong to the family of terpenes, volatile sulfur compounds, methoxypyrazines, and C13 norisoprenoids (Ebeler and Thorngate 2009; Padilla et al. 2016). The bound forms are not odorant compounds that on hydrolysis during fermentation are converted to odorant compounds. Enzymes, such as glycosidases, hydrolyze the nonvolatile glycosidic precursors and carbon-sulfurylases that release volatile thiols from nonaromatic cysteine bound conjugates (Gunata et al. 1998; Tominaga et al. 1998). The secondary aroma is contributed by esters, higher alcohols, volatile fatty acids, and, to a lesser extent, aldehydes (Padilla et al. 2016). Some of these compounds, like acetic acid, are responsible for the acidity of the wine, and its optimum concentration ranges from 0.2 to 0.7 g/L (Lambrechts and Pretorius 2000). Likewise, aromatic complexity is imparted by the group of aromatic compounds, higher alcohols below the level of 300 g/L (Rapp and Mandery 1986). The compound responsible for the fruity or floral aroma in wines is a group of volatile esters. Esters are both enzymatically and nonenzymatically produced from the preformed alcohols and acyl CoAs; however, the enzyme-free process contributes less to the final wine aroma; ethyl acetate is the most predominant ester formed during wine production derived from acetic acid and ethyl esters of saturated fatty acids; and at the level of 150–200 mg/L, it can lead to spoilage (Lambrechts and Pretorius 2000). However, isoamyl acetate, even though produced at low concentrations, is a significant contributor to aroma, mainly because of the lower flavor threshold as compared to the higher amount of ethyl acetate found in wine (Walker and Stewart 2016). Aldehydes, namely, acetaldehyde, confer apple-like fruity aroma to the wine, and the amount can vary from 10 mg/L up to 300 mg/L (Lambrechts and Pretorius 2000). A higher amount of acetaldehyde is produced by the *Saccharomyces* species (5–150 mg/L) than the non-*Saccharomyces* species (only up to 40 mg/L) (Fleet and Heard 1993). The high reactivity of acetaldehyde leads to the generation of the wide variety of other odor and flavor compounds, diacetyl being one of them (Lachenmeier and Sohnius 2008). The characteristic sensory property of the wine is also contributed by the process of malolactic fermentation (MLF). MLF or biological deacidification is a spontaneous process of conversion of malic acid to lactic acid and CO_2 carried out by lactic acid bacteria. Apart from the role of deacidification, MLF contributes to the flavor, aroma, and texture of the final wine product, by producing various metabolites like acids, alcohols, and esters (Izquierdo Canas et al. 2008; Lasik 2013).

11.3.1 Process of Winemaking

11.3.1.1 Selection of Grapes

The type and quality of grapes greatly influence the chemical composition of the wine. Grape variety in a particular region depends upon certain factors like biology, climatology, economics or traditions of that wine region (Hartmeier and Reiss 2002).

The primary aim of viticulture is to produce quality grapes that reflect varietal flavor and aroma in the wine. The ripeness stage during harvest is also crucial in the process of winemaking. The study showed that the ripening stage had a major impact on tannin extraction and astringency. With the ripening stage, the tannins from the skin and stem increased, whereas there was the decreased contribution from the seeds (del Llaudy et al. 2008). Similarly, another study showed that the color of the resulting wine was influenced by the grape variety. The wine color was determined in terms of PVPP (polyvinylpolypyrrolidone) index, spectrophotometric analysis, tint, total polyphenol index (TPI), total anthocyanins, and tannins (Perez-Lamela et al. 2007). Along with the grape cultivar, the postharvest techniques and fermentation methods further determine the sensory characteristics of wine (Styger et al. 2011). Harvesting by machines has increased, since it is more economical and time-saving than handpicking. However, handpicking is less damaging to the grapes and is still implemented for superior grape quality.

11.3.1.2 Crushing

Following harvest and after sorting and cleaning, the grapes are crushed and the must is used directly for fermentation in the case of red wine production or pressed to release the juice for producing white wine. The grape skin not only contributes to the color of red wines but is also often associated with the flavor. Fermentation of red wine is carried out on the skin to allow enough extraction of color compounds, like tannins and anthocyanin, as well as other flavoring compounds. In the case of white wines, before fermentation, pressing is carried out where the skins are removed. The final juice yield is influenced by the types of press used, like the screw press, basket press, moving head press, bladder or membrane press, impulse press, and belt press (Hartmeier and Reiss 2002).

11.3.1.3 Amelioration

After crushing the juice sugar, concentrations are determined by hydrometers by measuring the specific gravity of the juice (Boulton et al. 1995). The hydrometric scale used in measuring sugar content in juice is in terms of degree Brix and total acidity. One $^{\circ}$Bx means 1% (w/w) sugar. A sugar content of 22–24 $^{\circ}$B is considered suitable for preparing a wine of about 12% alcohol (v/v) (Kaur et al. 2019).

11.3.1.4 Starter Culture

During traditional winemaking, spontaneous fermentation of grapes occurs naturally by the native non-*Saccharomyces* species and lower population of *Saccharomyces* species. Industrial, large-scale wineries use specially selected starter cultures of *S. cerevisiae* strains available as active dry yeast, which shortens the lag phase and leads to rapid and complete fermentation (Bauer and Pretorius 2000). Occasionally, commercial non-*Saccharomyces* starter cultures are also employed in making wine with characteristic flavor and aroma. However, the trend has shifted toward using mixed starters containing one established *Saccharomyces* wine yeast along with non-*Saccharomyces* yeast (Walker and Stewart 2016). Yeast culture inoculums are prepared 2–3 days prior to the crushing of grapes. Fermentation starts within 24 h

and is marked by bubbling due to the growth activity of yeast, and this is used as inoculum within 2–3 days. Approximately, 200–500 ml inoculum volume is used for 10 L of must (Kaur et al. 2019).

11.3.1.5 Fermentation

Fermentation begins during the first phase of the *Saccharomyces* growth, when the cell number reaches 10^7 to 10^8 cells/ml and lasts between 2 and 5 days. It is during this phase that the fermentation occurs at a maximum rate and constantly consuming about a third and a half of the initial sugar content (Castor and Archer 1956).

The crucial metabolic pathway occurring in alcoholic fermentation is a catabolic pathway of transforming hexose sugars, particularly glucose and fructose, present in the must into ethanol and carbon dioxide (Aranda et al. 2011). Fermentation of wines is carried out in open or closed fermenters. Open fermenters have wooden headings to keep the cap submerged to enhance color extraction and further inhibition of oxidation processes (Hartmeier and Reiss 2002). Temperature is one of the relevant reliable variables that plays an important role during fermentation. Wine fermentation at lower temperatures of about 10–15 °C is employed to enhance production and retain flavor volatiles. Cryotolerant yeasts, such as *Saccharomyces bayanus* var. *uvarum* and *Saccharomyces kudriavzevii*, are generally used in low-temperature fermentation process (Lopez-Malo et al. 2013). White wines are usually produced at lower temperatures, and for the production of red wines, fermentation temperature of about 20–30 °C is used for about 7–14 days. Higher temperature contributes to the extraction of pigments from the grape skin (Kumar et al. 2008). The release of heat is important during fermentation, since the process is exothermic and leads to the death of yeasts (Williams 1982). Therefore, wineries employ large fermenters with automatic temperature control systems. The amount of sugar is also an important parameter to be monitored during fermentation, and it is controlled by measuring the sugar content by a Brix hydrometer or refractometer. As the fermentation proceeds, the frothing of the must occurs, which is characterized by the foam and skin that forms a "cap" at the top of the fermenting liquid. Industrially, mechanical separators are used to break the cap. Fermentation is carried out for till; the Brix reading is approximately 8, which takes about 4–5 days at ambient temperature and about 8–10 days at cooler temperatures (Kaur et al. 2019). Color extraction is 80% complete when the fermentation has progressed halfway (Hartmeier and Reiss 2002).

11.3.1.6 Malolactic Fermentation

Malolactic fermentation (MLF) is a secondary fermentation that occurs after alcoholic fermentation is carried out by a variety of lactic acid bacteria. It is essential for all red wines and some white wines, especially those undergoing bottle or barrel aging (Lonvaud-Funel 1995). It is a biological process of wine deacidification, where monocarboxylic L-lactic acid and carbon dioxide are formed from the dicarboxylic L-malic acid (Davis et al. 1985; Liu 2002).

This process is normally carried out by strains of *Lactobacillus, Leuconostoc, Oenococcus,* and *Pediococcus,* and they are resistant to low pH of less than 3.5, high

SO_2 around 50 ppm, and high ethanol levels of 10% v/v. However, the preferred species for MLF is *Oenococcus oeni*, because of its high acid tolerance and contribution to flavor (Liu 2002). Wine acidity is mainly contributed by malic acid and tartaric acid which comprises about 90% of the total organic acid found in wine. A very high concentration of malic acid, which may be 9 g/L, is found in grapes from cooler climates; hence, MLF is crucial in fermentation carried out in cooler countries (Lasik 2013). Apart from decreasing the total acidity of the wine, MLF also induces changes in the organoleptic character of the wine. The co-metabolism of glucose and citrate by *O. oeni* not only enhances bacterial growth rate and biomass but also enhances the formation of volatile acid-like acetate in wine, which can affect wine aroma and also the formation of carbonyl compounds mainly diacetyl that imparts buttery flavor. There is also a change in the color of the wine associated with the metabolic activity of bacteria on phenolic compounds (Lonvaud-Funel 1995).

11.3.1.7 Clarification and Fining

The wine produced after fermentation is cloudy mainly due to cell debris and other fermenting materials. The time-consuming type of clarification is the natural settlement of suspended particles at the bottom, after which the wine is carefully siphoned without disturbing the settled debris. This process is repeated until clear wine is obtained (Kaur et al. 2019). The traditional method of wine clarification is repeated decantation or filtration along with the help of fining agents (Reeves 1999). Fining is the process of adding adsorptive substances to the wine followed by settling or precipitation. PVPP (polyvinylpolypyrrolidone), casein, albumin, and gelatin are added to remove the wine astringency due to tannic acid or phenolics (Hartmeier and Reiss 2002). Fining agents, such as bentonite and celite, are used to make it sparkling clear, after which the wine is finally racked or filtered, if necessary, and bottled (Kaur et al. 2019) (Fig. 11.5).

11.3.1.8 Storage and Bottling

Aging is a process that occurs between the completion of fermentation and bottling of wine. Aging is a crucial process in wine technology that occurs in barrels, tanks, or bottles. Popularly, storage vessels are stainless steel tanks, wax-coated tanks, synthetically lined vessels, small and large wooden vats, and casks. Stainless steel tanks are used for normal storage, whereas wooden barrels are used to bring about chemical changes in wine, to lower astringency, and development of new complex aromas called "oak aromas" (Teodosiu et al. 2019). Aging wines in wooden barrels are however not cost- and time-efficient, so new technologies are focused on using oak chips as a valid alternative to reduce cost and providing wood flavored wine and less environmental impact (Garcia-Alcaraz et al. 2020). Similarly, electric field and ultrasound are new wine aging technologies. Ultrasound is known to reduce tannins, and the electric field enhances the extraction of phenolic compounds in wine (Garcia Martin and Sun 2013).

Clean bottles are used for bottling wine. Red wines are bottled in green or brown bottles. After filling, the bottles are corked using an automatic filling and corking machine (Kaur et al. 2019).

Fig. 11.5 Process of winemaking

11.3.2 Types of Wines

Wines mainly made from the grapes are still sweet or dry, either white, red, or rose wine, depending upon the color with 8–14% alcohol content. Dessert wines with a higher alcohol content of more than 14% are produced by fortifying with spirit or alcohol. Wines are fortified with herbs or spice mixtures to produce special wines called vermouths with 15–20% alcohol (Joshi and Attri 2005). Sparkling wine is obtained by the second fermentation of still wine called base wine. It is characterized by excess carbon dioxide, giving an effervescence, and contains about 10–12% alcohol content (Joshi and Attri 2005., Pozo-Bayon et al. 2009).

11.3.2.1 Production of Sparkling Wine

The distinct feature of sparkling wine as compared to still wine is the effervescence due to the presence of CO_2 bubbles, which are released when the bottle is uncorked (Pozo-Bayon et al. 2009). The cheapest way of producing sparkling wine is by carbonating it under high pressure. Another method is the secondary yeast fermentation of sugared wine that releases excess CO_2 (Hartmeier and Reiss 2002). The secondary fermentation can be carried out in a large hermetically closed vessel, containing base wine sweetened with sugar and subsequent addition of yeast that

carries out secondary fermentation, which takes around 20 days. After fermentation, the sparkling wine is clarified under pressure and bottled (Pozo-Bayon et al. 2009).

Another method is bottle fermentation, where the secondary fermentation and aging take place in the same bottle that reaches the market. The process is comparatively expensive and produces higher-quality sparkling wines like Champagne, Talento, and Cava, respectively, produced in France, Italy, and Spain (Pozo-Bayon et al. 2009). In this process, the bottle is filled with sweetened base wine and yeast, where fermentation occurs for over months and even years. Clarification is achieved by riddling the bottle in an inverted position, due to which the yeast and other debris are deposited on the cork, following which the bottles are frozen. Unlike the classical traditional bottle fermentation, the sparkling wines can be produced by transfer method, where the wine after secondary fermentation is transferred aseptically under pressure to another bottle after being clarified and filtered (Pozo-Bayon et al. 2009).

11.3.2.2 Production of Fortified Wine

Fortified or dessert wines are produced by increasing the alcohol concentration of the original wine having an alcohol content of about 11–16% and can be as low as 7%. The alcohol content is increased by adding high-strength distilled alcohol, where the alcohol content reaches around 19–21% (Joshi et al. 2017). Depending upon the variation in the process, sugar content, or added flavor, fortified wines can be classified into sherry, port, Madeira, and other liqueur wines.

Sherry wines are fortified up to 18–21% alcohol that can be sweet or dry. The process was developed in Spain, where velo de flor (flower veil) or simply plor yeasts grow on the surface of wine fortified up to 15% v/v ethanol and produce acetaldehyde from ethanol, which after reacting with other wine components leads to characteristic sherry flavor. Fino, oloroso, and amontillado are different types of sherry wines with 15%, 18–20%, and 16–18% alcohol content, respectively (Hartmeier and Reiss 2002; Kaur et al. 2019).

Port wines initially produced in Portugal are made from red grapes and fortified with brandy. Fermentation is stopped when the alcohol content reaches 4–6%. Port wines are characterized by high astringency due to high tannins released from grape skin (Hartmeier and Reiss 2002).

11.4 Production of Distilled Beverages or Liquors: Whiskey, Brandy, Rum, etc.

Compared to beer and wine, distilled beverages or liquors, such as whiskey, rum, brandy, etc., have a higher percentage of alcohol content and are different from beer and wine, because they have been developed by distilling fermented grains, fruits, or other fermented starchy substrates. The source of all distilled liquor begins with the process of fermentation in which yeasts convert sugar to ethyl alcohol. While fermentation results in the production of alcohol, the ethyl alcohol concentration is low, because if the alcohol buildup goes over 15%, the yeast cannot survive. The

alcohol becomes toxic to the cells at higher concentrations, and therefore, normal fermentation achieves an alcohol concentration of only 10–15%. To increase the concentration of alcohol, a process called distillation is used, where the liquid is changed to vapor state, by boiling and then condensing the vapor to get back to the liquid state. Distillation can be used to separate a mixture of liquids as long as the liquids have a noticeable difference in their boiling point. The boiling point of alcohol is around 78.5 °C, which is much lower than that of water (100 °C), so when distillation of the fermented liquid is done, the alcohol is first evaporated by boiling the liquid above 78.5 °C but below 100 °C. The vapor of alcohol is condensed by cooling and then collected. Only the alcohol can evaporate at this temperate but the water does not. Compared to the original fermented fluid, the distilled liquid now has a higher alcoholic content. This forms the basis for the production of distilled spirits or liquors.

11.4.1 Distillation Process

There are three major components required for the distillation process. First is the still or distillation flask, which is used to heat the liquid mixture into the gas state; the second component is the condenser, which cools the vapor back to the liquid state; and the last component is the collection vessel for collecting the condensed liquid. Based on this, there are two methods of distillation that are commonly used. The first is the pot still distillation and the other one is the column still distillation (Shipman and Thomas 2020).

Pot still distillation is the oldest and most common method of distillation used (Kockmann 2014). It consists of a large pot or kettle that is made with copper, which is heated at the bottom allowing the alcohol to evaporate into the vapor state. In the pot still, the alcohol and water vapor combines with ester that gives its aroma. The vapor is then passed into a coiled condenser, where it is cooled and then collected into a collection vessel. This process can be repeated to increase the concentration of alcohol. Pot still distillation is commonly used to distill liquors, for example, whisky, and operate on a batch distillation.

Column still distillation is a continuous still distillation, where the fermented liquid is fed continuously and the output streams, i.e., the distilled liquor are also removed continuously (Fig. 11.6.). It is made mostly of stainless steel and has two columns and is able to rectify the alcohol at a higher concentration by removing unwanted compounds. Column still is commonly used for vodka. The column is consisting of series of trays or plates. The first column is where the feed is supplied from top, down the tray, while hot steam is blasted from the bottom of the column. The steam volatilizes the alcohol in the feed and travels up to the top of the plates. As the alcohol moves to the top, its concentration keeps on increasing. In the second column, the alcohol is rectified as it condenses and circulates the column and condenses at the required concentration. Using column still, alcohol by volume ABV level as high as 96% or 192 proof can be achieved. Alcohol proof measures the ethanol content, e.g., 200 proof means 100% ethanol content. Most distilled

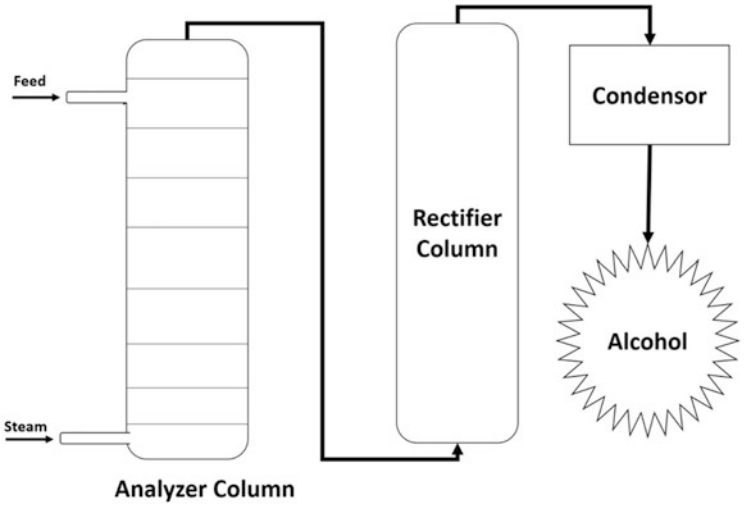

Fig. 11.6 Column still distillation

liquor has ethanol content close to an ABV of 40% or 80 proof. For most industrial process, column still is being preferred, but pot still distillation is common since it retains more flavor.

11.4.2 Aging and Maturation

Aging refers to storing the distilled liquor in barrels allowing the liquor to mature and acquire a unique color and flavor. These barrels are normally made of oaks, which impart tannins and other phenolics compounds to the liquor (Caldeira et al. 2008). Aging is affected by the nature of the wood, but it is also affected by the conditions of the cellar, where the liquor is aged. Conditions such as temperature and relative humidity play an important role in aging. Though aging provides a distinct flavor and taste to the liquor, depending upon the aging process, not all distilled liquors are aged. Distilled liquors that are commonly aged include whisky, rum, and brandies, whereas vodka and gins remain unaged (Fig. 11.7.). Vodka is an unaged colorless liquid, but unlike gin, it is non-flavored. Both are made from the distillation of fermented grains, but the gin has been flavored with juniper berries (*Juniperus communis).* The time required for aging also varies with single malt Scotch requiring a minimum of 3 years for it to be aged and classify as Scotch. Rums are normally aged in charred oak barrels. The period of aging varies from one liquor to another.

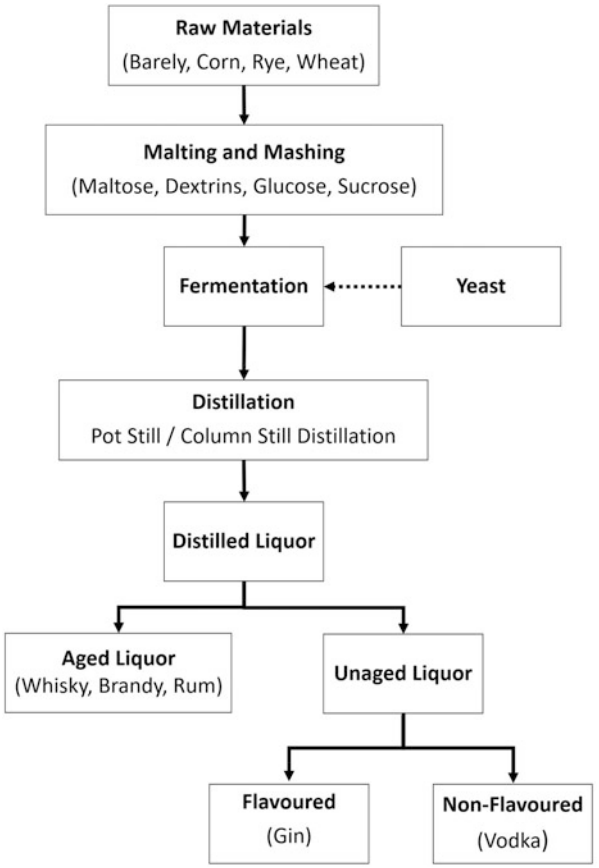

Fig. 11.7 Different steps in the production of distilled liquors

11.4.3 Whisky

Whisky is the most popular distilled liquor that is consumed globally. Whisky is derived from the distillation of fermented barley or cereals crops, such as corn, rye, and wheat. Whisky is commonly made from the fermentation of barley, but corn (maize) has also been used worldwide for making whisky. Rye grain is not very commonly used, since fermentation is less efficient, but the flavor imparts a characteristic flavor to the final product. Wheat, because of its high price, is not regularly used as a source of whisky. One of the most popular brands of whisky is Scotch whisky, which originated in Scotland and was originally called "uisce beatha" in Scottish Gaelic, which in Latin translation means "aqua vitae" or "water of life." Scotch whisky is made of malted barley or grain or a blend of the two. To be labeled as "Scotch whisky," the whisky must be produced in Scotland and can be single malt or single grain and must be matured in charred oak barrels for at least 3 years. Single malt Scotch whisky is made up of malted barley, water, and yeast in a single distillery using pot distillation, whereas single grain is made up of different grains,

such as corn, rye, or wheat, in a single distillery but using column distillation. Single-grain whisky is normally blended with malted whisky to give rise to blended Scotch whisky, which is the best-selling liquor and accounts for 90% of the Scotch whisky produced. Bourbon is produced in the United States of America, and it is made from the distillation of fermented grain corn and is aged in charred oak barrels. Different countries have their own rules and regulations for the production, labelling, and marketing of whisky, but the basic principle behind the production of whisky always involves malting, fermentation, distilling, aging, and finally bottling of the finished product.

11.4.3.1 Malting and Mashing

Malting forms the basis of whisky production, where barley is malted by soaking in the water, allowing it to germinate to produce enzymes that will degrade the starch into fermentable sugar, and then the germination is stopped by heating and drying the barley. During the mashing process, the malted barley is heated after being mixed with a grain, such as corn, rye, or wheat, and water. During this process, starch in the grain is broken down into fermentable sugar by enzymes, such as amylases or diastase, produced by the malted barley that can be utilized by the yeast, such as *Saccharomyces cerevisiae*, and can convert the fermentable sugar to ethyl alcohol (Dolan 1979). Diastase is mixture of enzymes that are secreted by the germinating embryo that are involved in the saccharification of the cereal starch into the fermentable sugar. The most common fermentable sugars are maltose, maltotriose, maltotetraose, dextrins, glucose, sucrose, and fructose (Palmer 1989). The enzymes required for the saccharification of starch to fermentable sugars are naturally present in the malted barley. In some saccharification processes, the use of the exogenous amylolytic enzyme has been employed. These are common for non-Scotch whiskies. The wort, which is the liquid that has been extracted from the mashing process that contains the fermentable sugar, will be used for the fermentation process. The principle behind malting is the same as that of beer.

11.4.3.2 Fermentation

Fermentation of the wort is achieved by the various strains of *Saccharomyces cerevisiae* that converts the fermentable sugars into alcohol and carbon dioxide. The liquid at the end of the fermentation is known as the wash, and this forms the base for the distillation process. The wash has an ABV of 8–10% before distillation. Lactic acid bacteria and *Leuconostoc* spp. are used to increase the acidity of the mash, and this mash can then be used by the yeast for its fermentation process (Plessis et al. 2004). Most of the fermentation can be done in open vats or closed vats made up of either copper or stainless steel. Fermentation is done under till; the alcohol percentage reaches as close to 10%. The optimal fermentation temperature of traditional brewing yeast is 25–35 °C; hence, the temperature during fermentation must not exceed 35 °C as it will lead to a decrease in the yield of ethanol (Abdel-Banat et al. 2010). Under optimal conditions, the fermentation process is completed within 2–3 days. During the fermentation process, it is not only ethyl alcohol that is produced, but other substances, such as acetaldehyde, esters, tannins, aldehydes, and

methanol, can also be present. These substances are called congeners and are responsible for the taste and aroma of the distilled alcohol. The amount of congeners present is crucial for the flavor of the liquor and can be removed by rectification, which is repeated or fractional distilling off the alcohol. Rectified spirits can increase the ABV level as high as 96% through the column distillation process.

11.4.3.3 Distillation

For Scotch malt whisky, the wort is distilled twice, sometimes thrice in copper pot stills. First, the wash is distilled in a wash still by heating it above 78.5 °C but below 100 °C, so the alcohol evaporates to the top which is then passed through a swan neck, and the vapor is then passed into a coiled condenser, where it is cooled and then collected into a collection vessel. The first distillation process produces "low wines" that have an ABV value of 25–30% but also contain unwanted compounds, so a second distillation is done in a second still called the spirit still. The liquor left over after first distillation is called pot ale or burnt ale and can be used for animal feed. In the second distillation process, three fractions are collected from the spirit still. The first fraction is called foreshots, which contains the most volatile compounds, such as methanol, ethyl esters, and acetaldehyde, and gives a pungent aroma. This fraction is undesirable and recycled back to the spirit still. The second is the potable spirit or the new make spirit, which is collected for making whisky. The third fraction is called feints, which contains the least volatile compounds, such as long-chain esters that cause turbidity. The feints are also recycled back to the spirit still along with the foreshots back to the spirit still with low wines for distillation (Fig. 11.8.).

The congeners present in the foreshots and feints contribute to the flavor of the final whisky that is produced. The residue left in the spirit still is called spent lees and is run to waste. After the second distillation, the distillate will now have an ABV of about 70–75% (Kaur et al. 2019). If a higher concentration is required, then a third distillation can be done.

11.4.3.4 Maturation

Whisky is stored in oak wood barrels or casks, which allow it to age and mature. While maturing, the whisky becomes smoother, gains flavor, and draws its color from the cask, it is stored in. Depending on the wood used for storage and the period of storage, it will greatly influence the flavor of the final whisky produced. The lignins and lactones of the wood contribute to the sweetness and creamy flavor of the whisky, while the tannins not only affect the color but also provide the bitterness in whisky. Whisky is aged in charred oak barrels that can be new or reused. For a whisky to be classified as bourbon according to the laws in the United States, the wood used for storage must be new charred, white oak. The time of storage also varies, but for Scotch whisky, a minimum of 3 years is required.

11.4.3.5 Blending and Bottling

To get the right flavor, a blended whisky is created, where different types of whisky have been blended along with neutral grain spirit (90–95% ABV), flavoring agents,

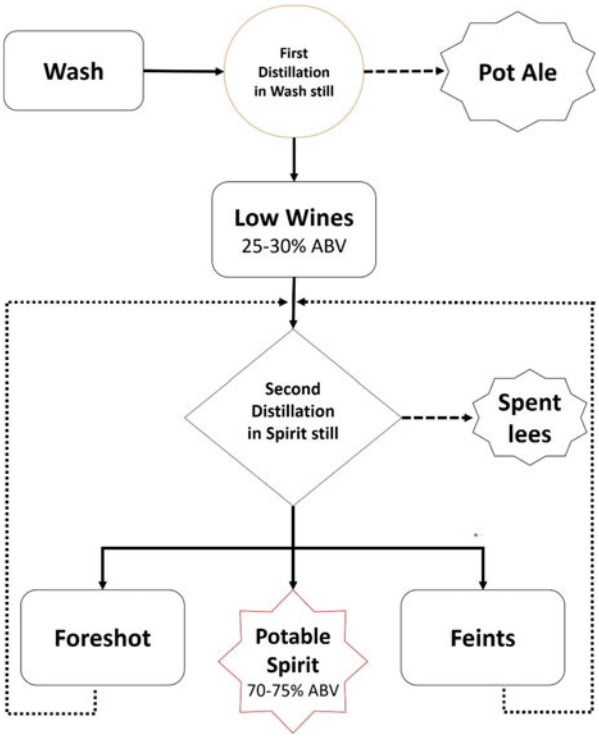

Fig. 11.8 Different stages in the double distillation of whisky

or color agents. Color of the whisky is achieved by aging but caramel may also be used, and flavoring agents such as sherry or port wine may be added to enhance the flavor of the blended whisky. Normally a high-quality malt whisky is mixed with a neutral grain spirit and other components. For example, blended Scotch whisky is made from a mixture of strongly flavored malt whisky and neutral grain whisky that is normally made from corn. Grain whisky is distilled in a column still while the malt whisky in pot stills. Single malt whisky is not blended and hence is made from a single batch and directly bottled. Bottling is done in glass bottle since whisky does not react or change in flavor in glass bottle. Most of the bottling have been automized for efficient production of the liquor.

11.4.4 Brandy

The term brandy comes from the Dutch word "brandewijn," which means burnt wine. It is derived from the fermentation of wines or any fruit. Brandy is most commonly made from the fermentation of grapefruits. The fermented juice or wine is then distilled twice to increase the alcohol content up to 35–60%, and then, this alcohol is aged in wooden oak barrels. In Latin, brandy is known as aqua vitae, while the French refer to brandy as "eau de vie," meaning water of life. The process of

brandy production is similar to that of whisky. There are different varieties of brandy produced throughout the world with Cognac and Armagnac being the most popular, owing to their unique flavor. These brandies are produced from the Southwestern region of France, with Cognac being produced from the Cognac region while Armagnac being produced from the ger districts of France.

Brandy is derived from the distillation of wine. Wine is normally derived from the fermentation of fruits, the most common being grapes, but apples, strawberries, raspberries apricot, cherries, etc. can also be used for the production of brandy. The quality and composition of brandies vary widely, as it depends on the raw material and the processing procedures used. Fermentation of the wine is done by pure starter cultures of *S. cerevisiae* (Walker and Stewart 2016).

Cognac is distilled twice in pot stills. Armagnac is distilled in a continuous column still. Double distillation increases the alcohol content of wine, say from 10% to 60% in the first distillation and then to 80% in the second distillation. Brandies are rich in volatiles as compared to other types of spirits, as it possessed high amount of alcohols and esters. The quality of distillates is greatly defined by their aromatic compounds, which appear during different stages of brandy production. These aromatics are mostly esters of fatty acids (Tesevic et al. 2005).

Brandies are also aged in oak or casks for 3 to 5 years; it can also be longer in some cases, till the color changes to golden or brown. This may take several months or several years. In Spain, brandy is aged using the solera system, where young brandy is mixed with older brandy and allowed to aged together. Solera system produces a consistent quality of brandy over time. Caramel coloring agent has also been used to deepen the color to amber for some brandies. Kirschwasser is an example of unaged colorless brandy made from the fermentation of morello cherries. To get the final product, the liquor from different casks is diluted with water to get the final alcohol concentration of 40% (v/v).

11.4.5 Rum

Rum is made by distillation of fermented sugarcane *(Saccharum officinarum)* or molasses. Rums are produced in the Caribbean and those countries which have a higher yield of sugarcane such as India. Fermentation of sugarcane is also achieved with *Saccharomyces cerevisiae*, and it is mainly responsible for the fermentation process, but other organisms, such as *Pichia*, *Candida, Kluyveromyces*, and *Schizosaccharomyces pombe*, also contribute to the fermentation processes (Schwan et al. 2001).

The raw material for the production of rum is sugarcane and molasses, which is a byproduct of sugarcane after the sugar has been crystallized. Most of the rum produced is from molasses, and this is the traditional rum/industrial rum which is darker. Agricultural rum is made from the distillation of fermented sugarcane, which is softer and more aromatic compared to traditional rum. *S. Cerevisiae* when used in fermentation produces a higher concentration of alcohol and fatty acid as compared to *S. pombe* due to a higher fermentation rate. But *S. pombe* produces more esters

compared to *S. Cerevisiae*, and hence the rum it creates is more aromatic (Fahrasmane and Ganou-Parfait 1998; Walker and Stewart 2016). Common bacteria, such as *Lactobacillus* species and *Propionibacterium* species, are significant when the bacteriological quality of the raw materials and of water is good and the aerobic phase is shortened. The *Lactobacillus* species consumes sugar and has an acidifying effect, since it produces lactic, acetic, and formic acid which can be esterified. These bacteria are also responsible for the enhancement of the organoleptic properties of rum due to the production of diacetyl and butanediol (Peynaud and Lafon-Lafourcade 1961; Jay 1982). *Propionibacterium* gives the rum a unique flavor due to the high concentration of propionic acid that it produces (Suomalainen 1975; Jounela-Eriksson 1978). For sugarcane-based rum, pot distillation is done, but for molasses-based rum, column distillation is preferred. Pot distillation results in heavy rum, since it has more congeners. The lesser the congeners, the lighter will be the flavor of the rum.

Rums are normally aged in charred oak barrels. The longer the aging process, the darker the color of the rum becomes. Dark rums are aged on charred oak barrels for a long time, while the light rums are aged in stainless steel tanks, resulting in colorless rum. The blending of rums allows different styles of rum to be created, and the blend is then diluted with water to give rum of 40% ABV, which is then filtered and bottled.

11.4.6 Vodka

Vodka is an unaged clear distilled liquor made from fermented cereal grain or vegetables. Its ABV ranges from 35% to 50%, so it is a high-proof liquor. Vodka is traditionally consumed without dilution with water or commonly used in cocktails mix. Historically, vodka was originally produced in Russia and Poland, but now it is produced worldwide. Vodka is produced from fermented grain, such as corn, rye, or wheat, or vegetables, like potatoes, soybeans, or sugar beets. Most of the high-quality vodkas are made from cereal grains such as wheat. Vegetables like potatoes contain high starch as compared to sugar; hence, to transform the starch to fermentable sugar, the potatoes are mashed with malt barley. The malt barley provides the necessary enzymes to convert starch to fermentable sugar. Fermentation is carried out by yeast *S. cerevisiae* in stainless steel vat to ensure the little flavor is imparted to the ethanol produced. Column still distillation is commonly used to produce vodka but pot stills can also be used. Pot stills impart flavor to the liquor, so the spirit is filtered through activated carbon to remove the flavor (Aylott 2016). The fine spirit obtained after distillation has 190 proof or 95% ABV, so it is diluted with water to make it drinkable, i.e., 80 proof or 40% ABV. Now the diluted liquor is ready to be bottled and shipped.

11.4.7 Gin

Gin is a colorless distilled liquor, but unlike vodka, it is flavored with juniper berries (Juniperus communis). The name gin is derived from the word genever (old English), genièvre (French), and jenever (Dutch), all of which are derivatives of the Latin word juniperus, meaning juniper. Gin is traditionally made from fermented grain, like barley, wheat, and then distilled. During the distillation process itself, juniper berries, spices, and herbs called botanicals are added that impart flavor to the alcohol, but the predominant flavor must be that of junipers to be qualified as gin. Other botanicals include orange peel, cardamom seeds, cumin, fennel, etc., which can add character and complexity to the gin (Riu-Aumatell 2012). Gin ABV range from 30% to 40%, in the United States, gin must have an ABV of at least 40%, whereas in Europe, the ABV of gin must be 37.5%.

Gins are produced by distilling the fermented grain or mash and then flavoring the alcohol with botanicals for flavoring. However, distilled gins are created by redistilling the alcohol a second time in the presence of botanicals. Compound gin is made by mixing the neutral spirit with the extracts or essence of juniper berries or other natural flavoring agents and then left to infuse without redistillation. One of the most popular gin styles is London dry gin and often considered as a benchmark for other gins. It has a flowery and aromatic flavor, and it is redistilled in the presence of junipers or other botanicals. No other artificial flavorings can be added after distillation in the case of London dry gins, which can be done in the case of distilled gins. The botanicals release essential oils when heated with the alcohol, or it can be suspended above the liquor and imparts flavor via vapor infusion (Aylott 2003). After distillation or redistillation, depending upon the type of gin being made, it is diluted with water to get the final product with the correct proof or ABV say 70 proof or 37.5% ABV.

11.5 Conclusion

Alcoholic beverages especially malt-based have been widely consumed throughout the world since ancient times, and most of the alcoholic beverages are based on the fermentation of cereal grains or fruits by yeast especially *Saccharomyces cerevisiae*. Depending upon their alcoholic contents, they can be categorized as beer, wine, and distilled liquor. Different regions have different preferences for the type of alcohol they consumed, but due to alcohol production being industrialized, it has now become a common beverage throughout the world. Alcohol, such as beer, wine, whisky, brandy, rum, etc., is already being produced on an industrial scale all over the world, and it makes up an important part of the economy of a country. Though alcohol production has been for thousands of years, there's a lot more to alcohol production than just conversion of sugar to alcohol: it involves a complex process that has to take into account the flavor, aroma, and even color of the finished product. This chapter gives an insight into the world of malt-based alcoholic beverages and their production.

Competing Interests All the authors declare that they have no competing interests.

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Biotransformation of Industrially Important Steroid Drug Precursors

12

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Abstract

Steroids are known to act as wonder drugs. The production of steroidal hormones and drugs rests mainly on sterols of plant origin, but most of them are in the less active form. The initial biotransformation of phytosterols includes cleavage of the side chain on the carbon-17 that brings 19 carbon key intermediates androst-4-ene-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD). Hydroxylations and dehydrogenations of nuclear carbon atoms have been the reactions of choice, which have been thoroughly investigated for the production of a wide range of steroidal drugs. The hydrophobic nature of steroidal substrates limits their bioavailability to microorganisms. Hence, crucial properties of steroids and approaches adapted to increase its bioavailability, like two-phase aqueous-organic systems, natural and synthetic emulsifiers, surfactants, and cloud point systems, are employed that improve the biotransformation of commercial steroid drug precursors.

Keywords

Androst-4-ene-3,17-dione · Androsta-1,4-diene-3,17-dione · Phytosterols · Bioavailability · Microorganisms · Steroid biotransformation

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_12

Abbreviations

16-DPA	16-dehydro pregnenolone acetate
9α-OH-AD	9α-hydroxyandrost-4-ene-3,17-dione
AD	Androst-4-ene-3,17-dione
ADD	Androsta-1,4-diene-3,17-dione
C/N	Carbon to nitrogen ratio
CSL	Corn steep liquor
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
h	Hour
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
IR	Infrared
min	Minute
mM	Millimolar
mol %	Mole percent
MTCC	Microbial-type culture collection
NCIM	National Collection of Industrial Microorganisms
NRRL	Northern Regional Research Laboratory
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PMBN	Polymyxin B nonapeptide
PPG	Polypropylene glycol
Psi	Pound per square inch
QTLC	Quantitative thin-layer chromatography
RF	Retention factor
Rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
sp.	Species
subsp.	Subspecies
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TX-100	Triton X-100
UV	Ultraviolet
v/v	Volume by volume

12.1 Introduction

Steroids are fat-soluble, unsaponifiable lipids, containing a "cyclopentano-perhydro-phenanthrene" nucleus comprised of four closed rings (Fig. 12.1). In 1949, cortisone showed a powerful anti-inflammatory activity, and hence, it was used in the

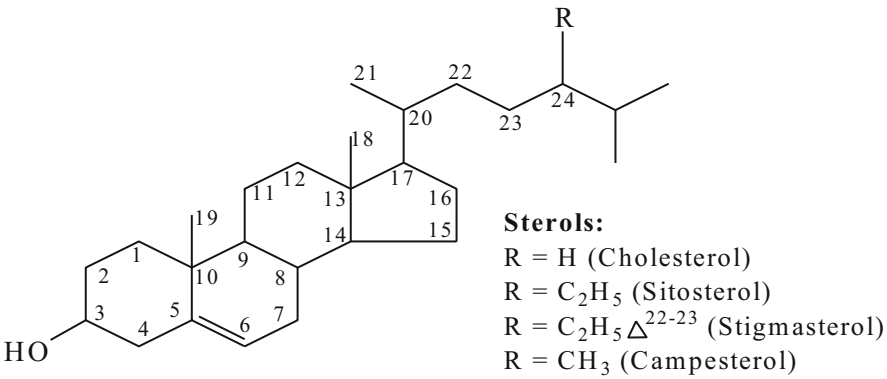

Fig. 12.1 Structure of steroid compounds (cholesterol, sitosterol, stigmasterol, and campesterol)

treatment of rheumatoid arthritis. Since then, several steroidal drugs bearing anabolic, anti-allergic, anti-inflammatory, cardiotonic, immunosuppressive, progestational, contraceptive, and geriatric activities are being used in modern medical practices (Martin 1984a, b; Miller 1985). Partial synthesis of metabolically active steroids from plants and animal sources offers several benefits in the production of steroidal drugs, through a combination of chemical and microbiological techniques. The steroid bioconversion work started in the 1950s when the pharmacological effects of progesterone and cortisone were known to the world. Microbial conversions of precursor steroids to either intermediates or final drug invariably involve fermentation step(s) using suitable microorganisms. In 1944, *Mycobacterium* sp. was reported to use phytosterols and cholesterol as carbon sources for growth and proliferation (Kieslich 1985). The selective 11α-hydroxylation of steroid compound by the fungus *Rhizopus arrhizus* proved to be an important phase in the development and production of commercially useful steroids possessing biological properties (Murray and Peterson 1952). Arima et al. (1969) reported that nearly 280 species of bacteria, actinomycetes, fungi, and yeasts could degrade >20% of cholesterol. Microbial conversion of steroids and sterols has been extensively studied for the synthesis of various sterol compounds having different hormonal or pharmaceutical properties.

Steroid hormones modulate gene expression via interacting with a variety of receptors present inside the cell, which act as transcription factors, and so, different aspects of cell proliferation and tissue differentiation are known to be regulated by these hormones (Rupprecht and Holsboer 1999; Baker 2011; Schüle et al. 2011). They have also been used to treat tumors of prostate and breast cancers as well as adrenal insufficiencies, by blocking aromatase (Johnston 1987; Banday et al. 2014; Gilbert et al. 2014), by inhibiting the production of cholesterol causing coronary heart diseases (Kutney et al. 1998). Steroids can also act as antiviral agents, e.g., torvoside H inhibits *herpes simplex virus* type 1 (Arthan et al. 2002); as antifungal agents, like azasteroids (Burbiel and Bracher 2003); as anti-obesity agents (Bays 2004); and as antibacterial agents (Abdelhalim et al. 2007). Some steroidal

medicines, such as raltegravir, inhibit the integrase of HIV and so prevent infection and are thus used to treat HIV infection (Dombrowski et al. 2000; Hicks and Gulick 2009).

The functions of neurosteroids are known, which improve the memory in mice (Shi et al. 2000; Markowski et al. 2001) and elicit the endocrine stress response, while, specifically, allopregnanolone acts as antidepressives and anxiolytic drugs (Schüle et al. 2011), corticosteroids, pregnenolone, and progesterone act as neuroprotective drugs (Garcia-Ovejero et al. 2005; Labombarda et al. 2013), diosgenin analogs as an anti-inflammatory agents (Singh et al. 2014), etiocholanolone as anticonvulsants (Zolkowska et al. 2014), etc. A steroidal drug dehydroepiandrostenedione (DHEA) helps to repair oxidative stress and mutagen-induced DNA damage in the brain (Shen et al. 2001). Topical steroids, corticosteroids, widely used for various skin infections include hydrocortisone and clobetasol, which prevent inflammation in response to several different stimuli. A major challenge in steroid biotransformation is the development of microbes for the specific transformation process. It is also called as fermentation process even though it does not involve the production of microbial metabolite as the final product. Steroids are the third-largest therapeutic agents, after the antibiotics and vitamins. Even today, steroid biotransformation is not only the process but also a state of the art, having several challenges related to its industrial production.

12.2 Steroid Production

The production of medicinally important steroidal moieties can be attained in two possible ways: either by total or partial chemical synthesis from the precursors which are found naturally. The chemical synthesis of steroid molecules demand expensive, multiple-step schemes, being complex molecules, which involve the creation of transitional derivatives with protected groups as well as the regeneration of such derivatives (Kolot 1982). Further, the basic ring structures of some steroid derivatives may get modified by various chemicals (Bensasson et al. 1999). The chemical synthesis also necessitates high temperatures and pressures as well as the use of potentially dangerous chemicals like sulfur trioxide or selenium dioxide and pyridine, which are hazardous to health and pose a harmful environmental issue of disposal (Weber et al. 1995a, b). Welzel et al. (1983) have identified a chemical route of androstenedione synthesis, by employing whole side-chain cleavage of cholesterol and phytosterols, but such chemical transformation involves lengthy procedures, including wary steps with lower yields and higher costs of chemical and harsh conditions (Fig. 12.2).

Contrary, on the other hand, the biological bioconversion of steroids is performed under mild to normal temperature and pressure conditions. Hence, biotransformation is a better alternative to chemical methodologies for the manufacturing processes of steroids.

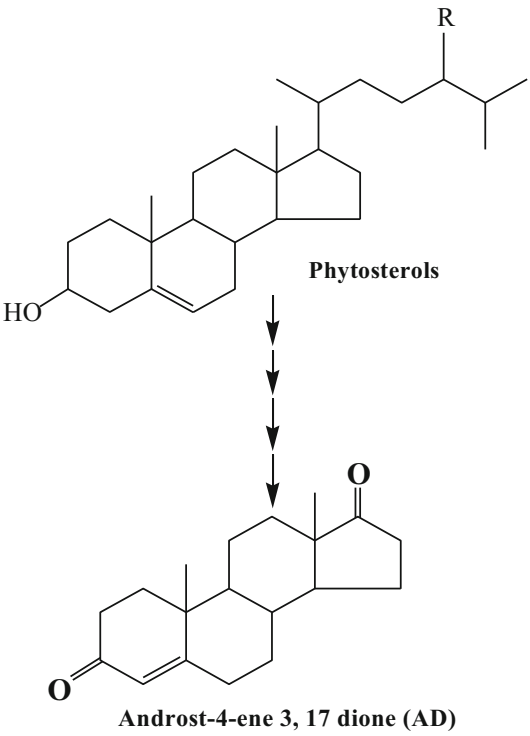

Fig. 12.2 Tedious and multistep chemical route for phytosterol to androst-4-ene-3,17-dione (AD) synthesis containing various steps at different conditions (Welzel et al. 1983)

12.3 Sterol Substrates

In the view of cutting down the cost of products, industries are always looking for cheaper and consistently available raw materials to produce steroidal compounds. Steroid from animal origin like cholesterol has been used to produce some products through the bioconversion (Chaudhari et al. 2010; Ahire et al. 2012), but it is not preferable because of formation of byproducts affecting the yield of the product. Phytosterols, like hecogenin, diosgenin, and solasodine, are traditional starting materials to produce pharmaceutically important steroidal drugs. Till 1970, sapogenins and diosgenin were the material of choice as the raw materials; however, the reduction in their availability increased its cost, making the related processes unprofitable. Now at industrial scales, other plant sterols are preferred substrates because of their higher availability and ease of transformation. Although several plant sterols are known, β-sitosterol, stigmasterol, and campesterol are available abundantly (Moreau et al. 2002; Fernandes and Cabral 2007). After the isolation of a *Mycobacterium* sp. that specifically degrades the carbon-17 side chains of phytosterols forming 17-ketosteroids, it became an essential starting material for the synthesis of steroidal intermediates like androstenedione (Marsheck et al. 1972; Pendharkar et al. 2014a).

For higher-scale production of phytosterols, vegetable and tall oil became the most important raw materials being available in more quantities (Coss et al. 2000; Hayes et al. 2002). Phytosterols can be extracted from plant sources, like soybeans (Perez et al. 2006) and bamboo shoots (Sarangthem and Singh 2003), and from low-cost sources, like sugar and paper industry waste (Perez et al. 1995; Dias et al. 2002; Perez et al. 2003). Chemical oxidation of stigmasterol usually yields progesterone, since the C22 double bond inhibits the basic degradation behavior of widely used microbial strains (Goswami et al. 1984). With 64% AD and 70% ADD yield, Perez et al. (2006) recorded successful bioconversion of soy sterols. A byproduct of soybean oil industries, viz. soybean extract, was employed as the raw material for the production of C17-ketosteroid (Donova et al. 2005). Dias et al. (2002) has recovered steroid intermediates from tall oil from Kraft process and paper mill waste with yields of approximately 42% β-sitosterol, 33% stigmasterol, and 25% campesterol are present in sugarcane press mud obtained from the filtration of sugarcane juice. It was used directly for the development of AD(D) using *Mycobacterium* sp. (Perez et al. 2006; Gulla et al. 2010). Metabolic pathways of β-sitosterol, cholesterol, campesterol, and their 3 β-acetoxy-19-hydroxycholest-5-ene and 19-hydroxy derivatives side-chain cleavages, using *Mycobacterium* sp., *Moraxella* sp., and *Rhodococcus* sp., have also been investigated (Szentirmai 1990; Murohisa and Iida 1993; Madyastha and Shankar 1994). Some uncommon phytosterols, lanosterol, lanosterol derivatives, and ergosterol, have also been used as substrates for steroid synthesis (Ambrus et al. 1995).

12.4 Steroid Reactions

There are mainly two mechanisms by which microbes convert these large and hydrophobic sterol substrates into steroid hormone or steroid drug precursors.

12.4.1 Side-Chain Cleavage

The foremost important reaction in steroid biotransformation is the side-chain cleavage of the sterol substrate, where phytosterols get converted into 17-ketosteroids are known as androsterones. Sih et al. (1968) classically explained how microorganisms degrade the C17 side chain of cholesterol to produce 17-ketosteroids. This process leads to the synthesis of two 17-ketosteroids, namely, AD and ADD. The sitosterol side-chain cleavage requires the regeneration of some cofactors, like $FADH_2$, NADH, and propionyl-SCoA. It starts with the attack of the sitosterol molecule's apolar end. In the terminal C27 methyl group, a hydroxyl group is added, which is then oxidized to a carbonyl group before being carboxylated at carbon number 28, which is enzyme-catalyzed and sitosterol-induced reactions. The first step was a group of three enzymes that could catalyze the reaction during the induction period at low partial oxygen tension by several saturated oligo-isoprene derivatives. The dissolved CO_2 concentration influences the second enzymatic step.

It was observed that propionate and, preferably, propionyl-SCoA could induce sitosterol side-chain cleavage by *Mycobacterium* sp. (Gyure et al. 1993). Many researchers have documented the bacterial metabolism of sterols and identified their metabolic intermediates (Sih et al. 1968; Owen et al. 1985). Androst-4-ene-3,17-dione is converted into androsta-1,4-diene-3,17-dione by Δ^1-dehydrogenationethyn reaction, where a double bond is introduced in the C1 and C2 of AD forming ADD.

This work resulted in the development of the chemical structure of androsterones and their relationship with other sterols. Both androsterones have been identified as vital intermediates necessary to produce androgens, estrogens, and anabolic drugs substantially used in allopathic medicine (Martin 1984a, b; Ahmad et al. 1991). The synthesis of the whole range of steroid drugs and their derivatives used as medicine is based on the availability of AD(D) and 9α-hydroxy-AD. Out of these three, AD is an indicative member of 17-ketosteroid family, because it is used for the manufacture of various derivatives of steroids, such as testosterone, testolactone, estradiol, ethinylestradiol, progesterone, prednisone, prednisolone, cortisone, and cortisol (Westfechtel and Behler 2006). This hormone is naturally present and produced in the gonad and ovary. In males, it is testosterone, while in females, estradiol and estrone are produced by using AD precursor. The most potent male and female sex hormones are testosterone and dihydrotestosterone (commonly androgen) and estradiol (estrogen), respectively. Arima et al. (1969) reported the development of AD by some species from the genera *Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Mycobacterium, Nocardia, Protoaminobacter,* and *Serratia* upon side-chain cleavage of sitosterol. When *Mycobacterium phlei* and *M. butyricum* were grown in a suitable medium containing some metal ions, like Co, Ni, Se, or Pb, it accumulated significant amounts of AD from cholesterol (Van der Waard et al. 1969).

Marsheck et al. (1972) described a microbial degradation method for cholesterol and plant sterols using *Mycobacterium* sp. NRRL B-3805 and *Mycobacterium* sp. NRRL B- 3683, to produce AD and ADD. A full pathway for the synthesis of AD by β-sitosterol biotransformation in *Pseudomonas* sp. NCIB 10590 was suggested by Owen et al. (1985). Szentirmai (1990) showed that 11 enzymes work together in 14 successive enzymatic steps, to degrade side chain of sitosterol to generate AD(D).

12.4.2 Nucleus Modification

Sterane nucleus modification is an important reaction that includes dehydrogenation, hydroxylation, and attachments of different functional groups (-CH3, -Br, -F) on the nucleus. The Δ^1-dehydrogenation introduces a double bond between the C1 and C2 that is observed in the conversion of AD into ADD, which typically could be achieved by using whole cells, since it relies on a cofactor and active cell machinery that continuously regenerates the necessary cofactors (Cruz et al. 2002). Dehydrogenation of C-C bonds within the steroid nucleus is a typical reaction carried out by organisms like *Actinoplanes, Arthrobacter, Corynebacterium, Nocardia,*

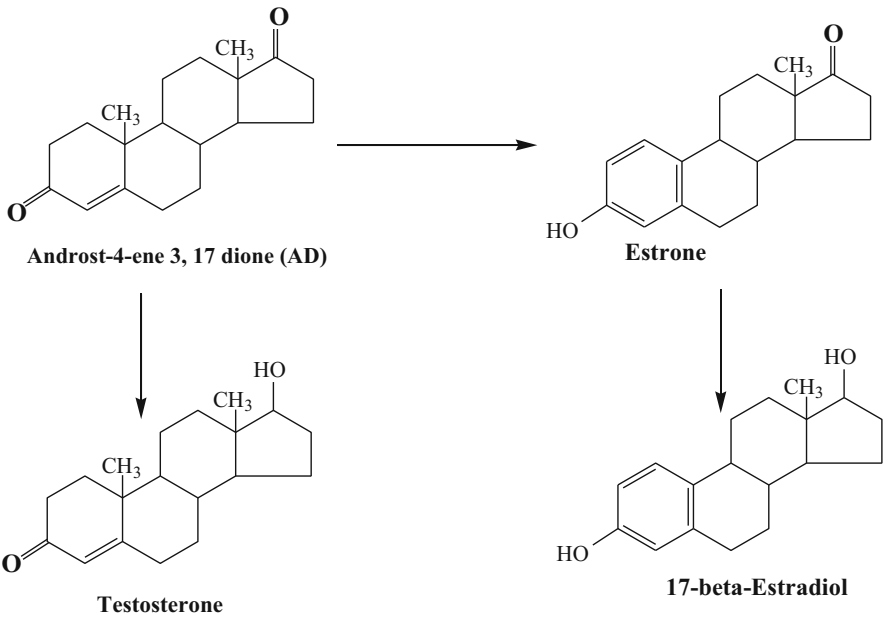

Fig. 12.3 Schematic flow of conversion of androstenedione to estrone, testosterone, and 17-β-estradiol (Westfechtel and Behler 2006)

Nocardioides, Micromonospora, Mycobacterium, Rhodococcus, and *Streptomyces*. Microbiological Δ^1-dehydrogenation of 3-ketosteroids is the foundation of technologies for manufacturing prednisolone and prednisteroids. *Arthrobacter simplex* is well reported for Δ^1dehydrogenation of androstanes, pregnanes, and derivatives of sterols and bile acids (Arinbasarova et al. 1996; Fokina et al. 2003). Higher level 3-ketosteroid-1(2)-dehydrogenase (3-KSD) activity was recorded in *A. simplex* (Sebek and Perlman 1979), *M. fortuitum* (Gulla et al. 2010; Pendharkar et al. 2014a), *Nocardia opaca*, *N. corallina* (Itagaki et al. 1990), *Rhodococcus erythropolis* SQ1 (Van-der-Geize et al. 2002), and *Nocardioides simplex* (Fokina et al. 2003), which is a key reaction in nucleus modification (Fig. 12.3).

Hydroxylation is one of the most common methods of bioconversion of steroids. Microorganisms that have an ability to hydroxylate sterol substrate at C1 to C21 and C26 have been identified by Mahato and Banerjee (1985); Manosroi et al. (1999). In the steroid industry, C7, C11, C14, C15, and C16 hydroxylation processes are presently established (Fig. 12.4) (Mahato and Garai 1997; Petrič et al. 2010; Wu et al. 2011; Kolet et al. 2014), owing to its demand for the development of adrenal cortex hormones and their analogs. Hydroxylation at carbon-9α is carried out by *Mycobacterium, Rhodococcus, Nocardia,* and *Corynebacterium* genera (Angelova et al. 1996), while 11α/β- and 16α-hydroxylations are usually catalyzed by *Aspergillus* sp., *Curvularia* sp., *Cunninghamella* sp., *Rhizopus* sp., and *Streptomyces*

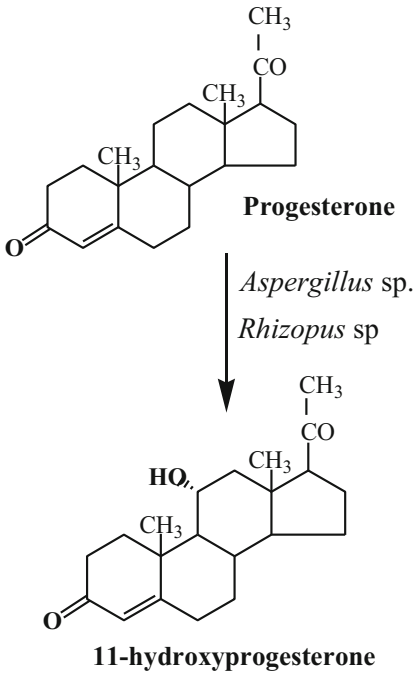

Fig. 12.4 Bioconversion of progesterone to 11α-hydroxyprogesterone by fungi (Petrič et al. 2010; Wu et al. 2011)

sp. (Mahato and Banerjee 1985; Sedlaczek 1988; Mahato and Mazumder 1995; Mahato and Garai 1997).

To exert anti-inflammatory action, oxygen must be present at position C11 of the steroidal ring, while glucocorticoid action is boosted by 16α-hydroxylated steroids (Sedlaczek 1988). Cultures of *Mycobacterium smegmatis* VKPM Ac 1552 and ATCC 12549 hydroxylate AD at position 14α, and so it is used for the synthesis of dihydroxy derivatives (Voishvillo et al. 2004).

The 9α-hydroxylation is catalyzed by ketosteroid 9-hydroxylase (9-KSH), which is comprising two ferredoxin proteins and a flavoprotein reductase as observed in *Nocardia* sp. M117 (Strijewski 1982). A useful intermediate is 9-hydroxy-AD, which is quickly dehydrated into the 9, 11-dehydro framework. Without the conventional 11-hydroxylation, it is possible to generate 11-keto structures as well as other 9α-halogenated corticoids. A small number of actinobacteria strains are known to conduct hydroxylation reactions at positions 1α, 2α, 12β, 15α, 15β, 17α, and 19, at low intensity. These hydroxy steroid compounds are further used for the synthesis of mono-, di-, or tri-hydroxyl derivatives of various active steroid drugs. Donova and Olga (2012) gave a detailed review on steroid transformation reactions, including chemical as well as biological synthesis processes.

12.5 Strain Isolation and Improvement

Several microorganisms having the ability to synthesize AD(D) have been screened out by several researchers. Microorganisms capable of degrading sterols, such as cholesterol or β-sitosterol, have been employed to promote efficient removal of side chains, leaving the sterol ring intact (Lee et al. 1997) (Fig. 12.5).

Manosroi et al. (1999) screened many microorganisms for 11β-hydroxylation and Δ^1-dehydrogenation. *Cunninghamella blakesleeana* SRP1 and *Bacillus sphaericus* SRP1 showed high 11β-hydroxylation and Δ^1-dehydrogenation activities, bringing a 62% yield of cortisol and a 67% prednisolone yield from cortexolone, respectively. Amid the crucial enzymes, KSH (3-ketosteroid 9α-hydroxylase) catalyzes the synthesis of 9-hydroxy-AD(D) from AD(D), and the expression of this enzyme is essential for the new strain development by genetic manipulation. Several KsdD or/and KSH mutants inactivated by treatment with UV irradiation and chemical mutagens or genetic engineering could generate metabolic intermediates that are

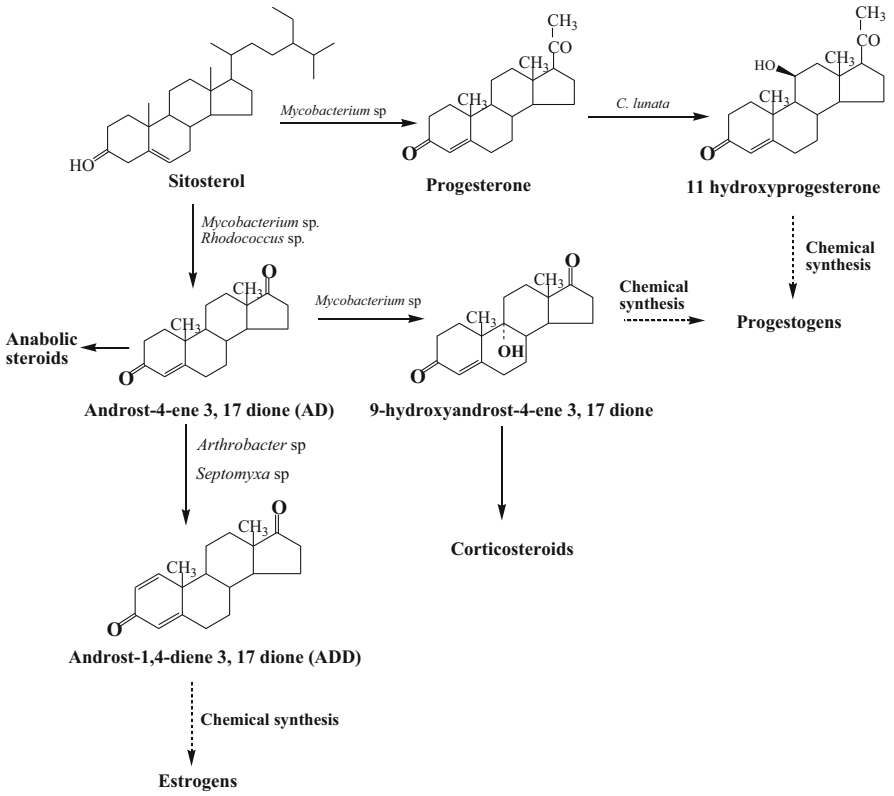

Fig. 12.5 Synthesis routes for major steroid intermediates through biological and chemical transformations (Mahato and Banerjee 1985; Owen et al. 1985; Mahato and Mazumder 1995; Angelova et al. 1996; Mahato and Garai 1997; Van-der-Geize et al. 2002; Fokina et al. 2003)

essentially required in the pharmaceutical industry. The resulting mutants lost the ability to expand on AD or ADD when one of the genes was deleted but retained the capacity to use 9-OH-AD as a substrate (Van-der-Geize et al. 2002; Donova et al. 2007).

Wei et al. (2010) isolated an actinomycete strain *Mycobacterium neoaurum* NwIB-01 from the soil, which can make use of phytosterols from soybean to synthesize both AD and ADD, where *KSH* gene was purified by using chromosome walking and nested-PCR. Yazdi et al. (1999) isolated *Agrobacterium* sp. M4 from the soil, having high potency of cholesterol degradation forming cholestenone as a byproduct. Bacillus strains HA-V6–3 and HA-V6–11 were isolated from the foregut of the Dytiscidae *Agabus affinis*. Both strains were capable to metabolize AD to yield 6β-OH-AD (Schaaf and Dettner 1998). A soil isolate *Micrococcus roseus* RJ6 was reported for bioconversion of cholesterol to AD and ADD (Dogra and Qazi 1999). The genes located on the plasmid express the enzymes involved in pathway for this conversion. Since sterol degradation property was lost after plasmid curing, Wadhwa and Smith (2000) used the culture enrichment method to screen out strains of *Bacillus sphaericu*s capable of progesterone bioconversion to synthesize AD from vegetable oil waste, where a high substrate concentration of sterol has been used. *Mycobacterium* sp. MB3683 and *Mycobacterium fortuitum* B-11045 were isolated, capable of using β-sitosterol as the sole carbon source that showed better biotransformation ability (Vidal et al. 2001).

The introduction of steroid conversion genes into a system that lacks steroid hydroxylation capability is a significant and attractive step forward, and many researchers have achieved it. Bovine cytochrome P450c17 was cloned and expressed in *E. coli* (Shet et al. 1997) and *S. cerevisiae* (Nishihara et al. 1997; Chen et al. 2020) with 17α-hydroxylation activity. In another cloning experiment, a steroid 9-α-hydroxylase gene from *Mycobacterium smegmatis* was transformed into *E. coli* BL2 by Arnell et al. (2007) for conversion of progesterone to obtain 9-α-hydroxyprogesterone. Similar enzyme activity was observed with 4-androstene-3,17-dione (AD) to produce 9-hydroxy-AD. Zhang et al. (2013) enhanced cortisone-to-prednisone bioconversion by gene modification in *A. simplex* 156, where extra copies of ksdD gene were added to the strain, which was regulated by a cat promoter from pXMJ19. Braun et al. (2012) inoculated *Yarrowia lipolytica* with human liver gene cytochromes P450 2D6 and 3A4 to improve bioconversion efficiency in a biphasic system for bioconversion of progesterone to 6β-hydroxyprogesterone and testosterone to 6β-hydroxytestosterone.

12.6 Modes of Substrate Addition

One of the most crucial aspects of steroid bioconversion is the lipophilic nature of sterol substrates in the aqueous medium, where microbe-mediated transformation is carried out. In water, the solubility of steroidal molecules is negligible, which is a bottleneck in its bioconversion. In aqueous conditions, the solubility of steroid and sterol molecules is typically less than 0.1 mM and 1 mM, respectively (Goetschel

and Bar 1992). To overcome this barrier, several strategies are employed to improve solubility of steroidal substrates in the aqueous incubation medium, where organisms are cultivated or grown. Some of the strategies include solubilization of the lipophilic substrates, by using organic solvents, surface active agents that help to improve the solubility, the addition of cyclodextrins, feed-batch addition of the inhibitory substrate, the addition of antibiotics to increase permeability across the cell wall to the broth, and in situ recovery of the inhibitory product.

12.6.1 Substrate Modification

The particulate lipophilic steroidal substrate that is added to the media tends to clump together, making dispersing it difficult. Hence, many researchers used microcrystalline and sonicated form of the substrate (Kondo and Masuo 1961; Maxon et al. 1966; Marsheck et al. 1972). Nondispersed and clumped substrate severely affects the solubility and ultimately reduces the final product conversion. Water-soluble derivatives or modifications of steroid substrate were also prepared, for example, cholesterol hemisuccinate (Kominek 1973) and polymethylsiloxane solution (Bhasin et al. 1976), for better bioconversion. The manufacturing steps of ether and carboxylic acid ester derivative of sterol have been patented by Westfechtel and Behler (2006). This derivative was biocompatible with water and readily emulsifiable. Therefore, it proves to be an effective raw material with higher yields for the manufacturing of AD(D).

12.6.2 Carrier Solvents

Buckland et al. (1975) reported the use of an organic solvent in steroid biotransformation for the first time, where *Nocardia* sp. was used to convert cholesterol to cholestenone in the presence of carbon tetrachloride. Later, it was found that, upon treating the cells with an organic solvent, it improves the membrane permeability, which subsequently improves the substrate and product movement throughout the cell membrane (Carrea et al. 1979). Chen and Whey (1990), while studying the hydroxylation reaction using *C. lunata*, suggested that the overall steroid biotransformation rate depends on the biocatalysts' particular behavior and the rate of hydrophobic solid substrate dissolution in an aqueous medium and concluded that mass transfer is the most crucial and a rate-limiting step in steroid biotransformations. Kutney et al. (2000) stated that by using micronized substrates, the mass transfer due to hydrophobic nature of sterol substrates can be improved moderately. Frequently, substrate steroids are dissolved in organic solvents, which are water miscible and added for biotransformation with enough grown biocatalyst cells. Some organic solvents that were reported are acetone (Lee et al. 1993; Srivastava and Patil 1995), dimethylformamide (Ghanem et al. 1992; Weber et al. 1995a, b; Patil et al. 2002), methanol (Berrie et al. 1999), 1,2-propanediol (Wang et al. 1998), dimethylsulfoxide, ethanol, hexane, toluene, etc. (Somal and Chopra

1982; El Refai and Abdel Salam 2010; Pendharkar et al. 2014a, b). Several other methods employ the two-phase system with water-immiscible solvents, including ethyl acetate, natural and synthetic oils, etc. (Arinbasarova and Koshcheyenko 1983; Ceen et al. 1987a, b; Phase and Patil 1994). Owing to these facts, it could be deduced that an organic solvent should allow maximum hydrophobic interactions while preserving the enzyme's native structure for an exhibition of its biocatalytic activity.

Ceen et al. (1987a, b) demonstrated that *A. ochraceus* uses highly hydrophobic fatty acids to hydroxylate progesterone at C-11. Flygare and Larsson (1989) used aqueous biphasic systems to study cholesterol side-chain cleavage by using polyethylene glycol (PEG), polypropylene glycol (PPG), polyvinyl pyrrolidone, and dextran (Gulla et al. 2010). Pinheiro and Cabral (1991) precisely investigated the toxicity of several organic solvents and solvent mixtures on steroid Δ^1-dehydrogenation activity of *Arthrobacter simplex*. These studies revealed that in systems with high proportions of organic solvents, both whole cells and pure enzymes can be deployed. However, the usage of such organic solvents poses some drawbacks, causing toxicity and catalytic deactivation at more than 5% concentration. The use of cyclodextrins has been very interesting and so thoroughly documented (Heselink et al. 1989; Donova et al. 2005; Donova et al. 2007; Wu et al. 2014). Cyclodextrins facilitate the solubilization of insoluble steroids by forming complex compounds that improve bioconversion. The use of cyclodextrins in phytosterol biotransformation by *Mycobacterium* sp. NRRL B-3683 boosted the yields of AD(D). In incubation medium, cyclodextrins prevent the sterol ring from splitting and therefore protects AD(D) by sequestration. Beta-cyclodextrin resulted in enhanced conversion yields than other types of cyclodextrins (α and γ) during sitosterol biotransformation to AD (Heselink et al. 1989).

12.6.3 Emulsified Substrate

Other strategies to address the issue of sterols' high hydrophobicity for the biotransformation comprises the use of surface-active agents for making substrate suspensions. Surfactants and emulsifiers, both synthetic and natural, were used as surface-active agents (Vadalkar et al. 1980; Vlavov et al. 1990; Mao et al. 2018). Martin (1977) used nonionic surfactants like tweens (polysorbates) and spans (sorbitan esters) as emulsifiers in biotransformation. Marsheck et al. (1972) bioconverted powdered sterol substrate into AD and ADD in a warm aqueous solution of Tween 80. Vadalkar et al. (1980) and Vlavov et al. (1990) exhibited the effect of different tween derivatives, including Tween 80 and Tween 40, 60, and 80, respectively, on the 1(2)-dehydrogenation rate of steroid molecules. The provision of fatty acid components is key to surface-active cell lipid biosynthesis. This enhances the effect of Tweens 40 and 60 on β-sitosterol co-oxidation mediated by *Nocardia* M 29–40. Tweens exhibit higher conversion by enhancing the mobility of lipophilic components, and not due to improving the dispersion of substrate (Schömer and Wagner 1980). Smith et al. (1993) successfully biotransformed cholesterol into 4-cholestenone, AD(D), testosterone, and 1-dehydrotestosterone

(DHT) in Tween 80 medium. Since then, Tween 80 (Perez et al. 1995; Perez et al. 2005), Span 80, and Triton X-100 (Vasudevan and Zhou 1997) have been employed to disperse the phytosterols in the medium. For the first time, Phase and Patil (1994) demonstrated the use of natural oils as carriers for the bioconversion of soy sterols to 17-ketosteroids by *Mycobacterium fortuitum* NRRL B-8153, which showed better conversion than in the presence of organic solvents. Using *Mycobacterium* sp. MB 3683, Kutney et al. (2000) and Stefanov et al. (2006) employed suitable solubilizing agents, such as PPG and silicone oil, to solubilize phytosterol concentrations up to 30 g/l in the nutritional medium.

When dried yolk was added to the medium, Imada et al. (1983) noticed an increase in the accumulation of ADD. Lee et al. (1993) used various emulsifiers, like silicon oil, Span 60, Tween 80, Triton X-100, and lecithin, for the bioconversion of cholesterol to androstanes. Rumijowska et al. (1997) found that lecithin had a favorable impact on the cell membrane *of Mycobacterium vaccae*, increasing cholesterol solubility and ultimately biotransformation at an appropriate concentration. By creating liposome similar structures with sterol particles, lecithin improves sterol solubility. For most microorganisms, lecithin is biocompatible and nontoxic, because lecithin and its derivative-like compounds are present naturally in the cell membrane. To make a stable colloidal solution of cholesterol in water with higher concentrations, a small amount of lecithin could be used. Therefore, incorporation of lecithin into the aqueous medium boosted the bioconversion both in terms of productivity and stability (Zhi et al. 2002).

Lee and Liu (1992) demonstrated the increased bioconversion of sterols to 17-ketosteroids by trapping the products in water-soluble resins like Amberlite XAD-2. While by adding corn flour and soybean flour in the biotransformation medium, an increase in phytosterols to AD transformation was recorded through the culture *Fusarium moniliforme* Sheld (Lin et al. 2009), where emulsification could be the possible reason.

12.7 Immobilization

The preferred and commonly used method for steroid biotransformation is whole-cell immobilization, since it offers many advantages over the use of a pure enzyme. It is cost-effective and reduces the inhibitory effects of the substrate. In a recurrent batch with 1000 mg/l cholesterol, the productivity of 190 mg/l per day with a 77% molar conversion of ADD was accomplished by immobilized *Mycobacterium* sp. NRRL B-3683 cells, where an activated alumina carrier was utilized for the immobilization of *Mycobacterium* sp. NRRL B-3683 cells, having 45 days half-life period (Lee and Liu 1992). For the two-step conversion of Rechstein's compound S (cortexolone) to prednisolone, immobilized co-cultures of *Curvularia lunata* spores and *Mycobacterium smegmatis* cells were used in a single-stage fermentation by Ghanem et al. (1992). For the 11α-hydroxylation of progesterone, polyurea-coated alginate beads of *A. ochraceus* cells employed in the organic and aqueous biphasic

system to overcome hydrophobicity sterol-based limitations of mass transfer in an aqueous medium (Dias et al. 1994).

Some carriers are employed to immobilize microbial cells having the ability to cleave phytosterol side chain. Table 12.1. depicts some of the immobilization methods as well as some process modifications. Amin et al. (2010) immobilized cells of *Mycobacterium* sp. NRRL B-3805 onto polyvinyl alcohol (PVA)−/polyvinyl pyrrolidone (PVP)-mixed polymer hydrogel. The highest AD yield was observed using the composition copolymer PVA/PVP (8:2, w/w), polymerized at a dose of 20 kGy γ-irradiation. Immobilized cells with PVA/PVP copolymer obtained a yield like that of free cells in 24 h of incubation. This immobilization of mycobacteria was further successfully run for ten cycles of biotransformation with a maximum (82%) yield and was higher than free cells (76%). In another report, Bou et al. (2013) used the *Luffa cylindrica* fruits to immobilize cells of *Mycobacterium* sp., where the cells got stuck into the dried fruits, and the interesting feature was that the cholesterol was not dissolved in any of the solvents.

12.8 Problems Associated with Steroid Transformations

The solubility of steroidal compounds is a major problem in steroid biotransformation that continues with low to moderate yields in general. Substrate carriers are effectively established now in steroid bioconversion industries. Different carrier solvents, like acetone, ethanol, ethyl acetate, and DMSO; emulsifiers like ethylene glycol, and PEG; surfactants like Tween (20, 40, 60, and 80), Triton X-100, and oils from sunflower, soybean, and silicone are being used to improve the solubility of the steroid and ultimately the product yield.

These reactions are always very simple having no requirement of any tedious protocol for biotransformation, and specific care is also not required. Many of the times, maintenance of sterility also may not be an essential requirement, even though it is a fermentation process involving microorganisms.

Immobilization of microorganisms is a method of choice in these processes, as it reduces the inhibitory effects of substrates as well as the toxicity of the products formed. Substrate and product, both exert some toxic effects if added in higher concentration. However, these toxic effects are reduced by immobilization of either organisms or the enzyme and by adding substrate in the feed-batch process. Recyclability using immobilized microorganisms could be very well achieved for sustained use of the culture or the enzyme, which also reduces the time required for cell growth. Overall, cost-effectiveness is essentially required in any industrial process, which is achieved in the biotransformation process when compared to chemical conversion. Generally, the biotransformation process is a single-step reaction while on the other hand, the chemical process is always a series of multistep reactions. Further, the cost of the process also depends on downstream processing; however, bioconversion does not require highly sophisticated and specific equipment for the separation of the product, making biotransformation a method of choice for the synthesis of pharmaceutically active steroid drugs or intermediates.

Table 12.1 New approaches used in the biotransformation of steroids with process modifications

Substrate	Organisms	Product	Process modification	References
Progesterone	*Streptomyces roseochromoges*	16α-Hydroxyprogesterone	Substrate dissolved in ethanol (20 mg/ml)	Berrie et al. (1999)
Phytosterol (from soybean oil)	*Mycobacterium* sp. MB 3683	AD and ADD	Polypropylene glycol and silicone	Kutney et al. (2000)
Progesterone	*Bacillus sphaericus*	AD and ADD	Glucose optimization; degradation of progesterone side chain; ethanol (0.05% w/v)	Wadhwa and Smith (2000)
Cholesterol	*Lactobacillus bulgaricus*	Testosterone	Glucose-controlled fermentation with cyclodextrin	Kumar et al. (2001)
β-Sitosterol	*Mycobacterium* sp. NRRL B-3805	AD	Celite used for cells immobilization	Llanes et al. (2001)
β-Sitosterol	*Mycobacterium* sp. MB-3683 and *mycobacterium fortuitum* B-11045	AD and ADD	Selection of high capacity transforming strain	Vidal et al. (2001)
3β-Acetoxypregna-5,16-diene-20-one (16-DPA)	*Pseudomonas diminuta* and *Comamonas acidovorans*	AD and ADD	Mixed culture; dimethylformamide (1%)	Patil et al. (2002)
β-Sitosterol	*Mycobacterium* sp. NRRL B-3805	AD	Two-phase system (diisoheptyl, bis- and di-isodecyl, and bis phthalates)	Cruz et al. (2002)
Cholesterol	*Mycobacterium* sp. NRR L B-36 83	AD and ADD	Lecithin (< 0.1%)	Wang et al. (2002)
β-Sitosterol	*Bacillus subtilis*	ADD	Fermented succulent bamboo shoots employed for β-sitosterol	Sarangthem and Singh (2003)
Cholesterol	*Mycobacterium* sp. NRRL B 3683	AD and ADD	Cloud point system	Wang et al. (2004, 2008)
Soybean scum and tall oil sitosterol	*Mycobacterium* sp. VKM ac-1817D	9-OH-AD	Substrate extracted from soybean oil mill waste, tween 80 (0.05%)	Donova et al. (2005)

Sugarcane mud	*Mycobacterium* sp. NRRL-B3683 and MB3683	AD and ADD	Sugarcane mud; tween 80 (1%)	Perez et al. (2005)
Cholesterol	*Fusarium solani*	AD and ADD	Corn steep solids used for medium modification, change in pH, and use of 8-hydroxyquinoline and methanol	Sallam et al. (2005)
β-Sitosterol	*Mycobacterium* sp. NRRL B-3805	AD	Chrysotile (asbestos) employed for cell immobilization; use of biphasic system	Wendhausen et al. (2005)
Isolated from soybean varieties VN1, VN2, VN3	*Mycobacterium* sp. NRRL-B3683 and MB3683	AD and ADD	New substrates VN1, VN2, and VN3; tween 80 (0.1%)	Perez et al. (2006)
Phytosterols	*Mycobacterium* sp. MB3683	AD and ADD	Two-phase water-oil system	Stefanov et al. (2006)
β-Sitosterol	*Mycobacterium* sp. VKM ac-1816D	AD and ADD	Modified β-cyclodextrins	Donova et al. (2007)
Progesterone and AD	Recombinant strain *E-coli* BL21	9α-OH-progesterone and 9α-OH-AD	9α-hydroxylase gene cloned into *E. coli* BL21	Arnell et al. (2007)
β-Sitosterol	*Mycobacterium* sp. NRRL B-3805	AD	Silicone rubber employed cell immobilization	Claudino et al. (2008)
β-Sitosterol	*Mycobacterium* sp. DSM 2966	AD	Microemulsion of nutrient broth / PEG200/triton X114/tween 80 / chloroform	Malviya and Gomes (2008)
Corn flour and soybean flour	*Fusarium moniliforme* Sheld	AD	Corn and soybean flour as substrate	Lin et al. (2009)
Soybean phytosterols	*Mycobacterium neoaurum*	AD and ADD	Cloning of enzyme gene (KSH) for hydroxylation	Wei et al. (2010)
β-Sitosterol	*Fusarium solani* NRC 105	AD and ADD	Combination of organic-aqueous phases	El Refai and Abdel Salam (2010)

(continued)

Table 12.1 (continued)

Substrate	Organisms	Product	Process modification	References
β-Sitosterol	*Mycobacterium* sp. NRRL B-3805	AD, ADD, and testosterone	Immobilization of cell PVA/PVP hydrogels	Amin et al. (2010)
Progesterone and testosterone	*Rhizopus oryzae* RA99880, *saccharomyces pombe* 1445	11α-OH-progesterone, 11α-OH-testosterone	Expression 11β-steroid hydroxylase in recombinant fission yeast	Petrič et al. (2010)
Cholesterol	*Chryseobacterium gleum*	ADD	Growing cells	Chaudhari et al. (2010)
Cholesterol	*Gordonia neofelifaecis* NRRL B-59395	ADD	Inhibitors of sterol nucleus cleavage, tween 80 (0.2%)	Liu et al. (2011)
16α,17-Epoxyprogesterone	*Rhizopus nigricans* AS 3.39	11α-OH 16α,17-epoxyprogesterone	Two ionic liquids phase	Wu et al. (2011)
Phytosterols and cholesterol	*Nocardia* sp.	AD and ADD	Inhibitors of sterol nucleus cleavage, tween 80 (0.2%)	Sharma et al. (2012)
Progesterone	*Yarrowia lipolytica* H222-S4	6β-Hydroxyprogesterone	Human P450 genes expressing recombinant yeast; two-phase system	Braun et al. (2012)
Phytosterols	*Mycobacterium* sp. DSM 2966	AD and ADD	*Luffa cylindrica* dried fruits employed for cell immobilization	Bou et al. (2013)
Cortisone acetate	*Arthrobacter simplex* M158	Prednisone acetate	Strain modification by transferring additional copies of *ksdD* gene.	Zhang et al. (2013)
Testosterone and progesterone	*Mucor hiemalis*	14α-Hydroxy-testosteone, 14α-hydroxy-4-androstene-3,17-dione, 14α-hydroxy-progesterone, 7α,14α-dihydroxy-progesterone	14α-hydroxylase containing cell-free extract	Kolet et al. (2014)
Dehydroepiandrosterone (DHEA)	*Colletotrichum lini* ST-1	7α,15α-Dihydroxy-DHEA	Cyclodextrin-substrate inclusion complex	Wu et al. (2014)
Phytosterol	*Mycobacterium neoaurum*	AD	Modification of methylcitrate cycle pathway by gene cloning	Zhang et al. (2020)

12.9 Conclusion

Since 1945, steroid fermentation is being used as a tool for the production of pharmaceutically active steroid compounds. This tool is emerging with newer technologies. A major portion of the pharmaceutical market is occupied by steroidal drugs. There is always a demand for newer and more effective drugs in the therapeutic market. However, the low yield of these compounds causes limited supply and an increase in cost. Newer technologies, like media formulations for fermentation, gene manipulations of strains, and substrate modifications, proved to be beneficial for increasing the productivity of these drugs. Immobilization of enzymes or culture in a single or mixed state is useful to uplift the current biotransformation processes. Isolation of newer microorganisms, possibly from the extreme environment which can tolerate a higher concentration of sterol substrates with high conversion efficiency, is a new requirement. Genetic modification like DNA recombination in microbial strains can certainly help to enhance the degree of bioconversion.

12.10 Future Perspectives

Increasing interest in the biotransformation of steroid compounds resulted in the discovery of novel pharmacologically active compounds. Newer technologies like the cloud point system, trapping of the product formed to avoid its toxicity on microbial cells, and use of carrier solvents are being developed that may help to enhance the current biotransformation scenario. In addition to the emerging new strains with genetic modification, there is also an enhancement in the availability of immobilized enzymes and the deceived culture media.

Acknowledgments GBP is thankful to the Principal, SGM College, Karad. TB and SPP are thankful to DAU for providing facilities for research work, and KSD and BLC are thankful to UGC-SAP program and DST-FIST program for providing facilities through KBCNMU.

Declaration All the authors declare that they do not have any competing interests.

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Value Addition to Chemical Compounds Through Biotransformation

13

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Abstract

In the last few decades, the production of value-added chemical compounds through biocatalysis using microbes or their enzymes is offering the development of environment-friendly and economically attractive processes. Owing to concerns over environmental pollution, sustainable transformations using enzymes or whole cells are being explored, since it is safer and environment-friendly than conventional chemical processes. Biotransformation process has proved to be an efficient alternative to widely accepted and practiced synthetic chemical routes, because of the unique properties of biocatalysts, namely, their stereo-, chemo-, and regioselectivity, resulting in a better quality of compounds and their capability to carry out reactions avoiding extremes of physicochemical parameters, like pH, temperature, and pressure. Although biocatalysis or biotransformation exhibits some lacunae in processes to produce chemicals, it fulfills many key criteria of green chemistry. Hence, biotransformation processes are being praised and being explored for the synthesis of chemical compounds on a laboratory scale and even on a large industrial scale. This chapter focuses on several industrial processes employing microorganisms or their enzymes for the synthesis of valuable compounds.

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_13

Keywords

Biotransformation · Biocatalysis · Value addition · Environmentally-friendly reactions · Green chemistry · Enzymes · Chemicals

Abbreviations

ADD	Androsta1,4-diene-3,17-dione
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
Cyt P-450	Cytochrome P-450
DHEA	Dehydroepiandrosterone
DOPA	3,4-Dihydroxyphenyl alanine
e.e.	Enantiomeric excess
HMF	5-Hydroxymethylfurfural
HPOPS	(R)-2-(4-hydroxyphenoxy)propionic acid
POPS	(R)-2-phenoxypropionic acids
TTN	Total turnover number

13.1 Introduction

The last two centuries are earmarked with tremendous developments upon discoveries and inventions that lead to bringing ease to human life. Cumulatively, these burgeoning acts through research and development and subsequent large-scale industrial production lead to serious negative environmental implications. Biological processes being sustainable and environment-friendly are being used to produce fuels, fine chemicals, agrochemicals, food, and pharmaceutical ingredients and hence are widely accepted. Generally, the conventional chemical synthesis processes are being used in pursue of high yields but are not environment-friendly, which has high potential to produce undesired by-products or racemic compounds which reduce productivity, leading to increased downstream costs. Biological processes comprising biocatalysts provide several benefits like its working at normal conditions, like temperature and pH, use of renewable resources, ease of operations, and high selectivity in both substrate and product stereochemistry (Jemli et al. 2016). Over the few past years, it was discovered that living microorganisms through their cascade of enzymes could modify many chemical compounds by single-step chemical reactions called "biotransformations." According to the European legislation and FDA, products having natural origin and modified through a biotechnological process could be considered natural (Xu et al. 2007). Biocatalysis has been more valuable than traditional chemical synthesis methods (Wachtmeister and Rother 2016). Microorganisms possess the capability to modify several organic compounds through the enzymatic route, which is known as biotransformation or bioconversions

or microbial transformations. The microbes are better known for their activity to ferment various substrates to produce the products over ancient times. Alcohol production has been a classical example of fermentation. The fermentation and biotransformation processes differ in the steps involved, where fermentation may have several catalytic stages, while biotransformation involves either one or two steps (Vasic-Racki 2006; Schrewe et al. 2013). Microbial biotransformation is regarded as a new tool for modifying the chemical structures of substrates with physical-chemical characteristics and better activities (Cano-Flores et al. 2020). Interestingly, there are several microorganisms, including bacteria, fungi, and algae, that are present all over the ecosystems, including the diverse environmental niches at an uncountable number having a great potential to act as catalysts in biotransformation processes. There are several microbes typically being used as factories for biotransformations including eubacteria, such as *Escherichia* sp., *Pseudomonas* sp. *Arthrobacter* sp., *Rhodococcus* sp., *Lactobacillus* sp., and *Saccharomyces cerevisiae* (as well as fungi such as *Aspergillus* sp. and *Mucor* sp.). When compared with chemical synthesis, biotransformation poses unique advantages like *stereo-* and *regio*selectivity that has the potential to produce chiral products (Leresche and Meyer 2006). Microorganisms show a broad substrate range and high selectivity, making them perfect biocatalysts for the synthesis of various industrially important chemical compounds (Straathof et al. 2002; Pollard and Woodley 2007). Biotransformation is finding more applications in the field of fine chemicals, cosmetics, pharmaceuticals, foods, and biofuels (Wiltschi et al. 2020). It is reported that about 85–90% of all chemical processes in the world are carried out through catalysis (Thomas and Harris 2016; Wittcoff et al. 2012), while estimated annual sales of chemicals were about 3500 billion USD in 2018 (Faber 2018). It is imperative that the biocatalytic processes are being used extensively to produce chemicals (Hauer 2020). Although hundreds of biotransformations have been reported, only a few of them can be used to synthesize commercial products. The biocatalysts pose drawbacks like a synthesis of the product accompanied with by-products, where cell wall and/or membrane acts as the barrier in its transport. Low yields lead to increase costs. However, a biocatalyst can certainly be modified to improve its performance through and metabolic engineering leading to the modified protein that helps to overcome the limitations. Nonetheless, chemical synthesis processes remain the processes of choice for several products. Hence, a multipronged approach for the improvement of the biotransformation process is essentially required (Lin and Tao 2017). In the biotransformation reaction, the conversion time required is dependent on the ability of the microorganism under use, the type of reaction, and the substrates being used. A variety of microorganisms are being explored and being tried to carry out various biotransformation reactions, which include actively growing organisms, resting cells whose growth is seized, the killer cells having their enzyme system intact, immobilized cells or enzymes which are trapped in some solid carrier, cell-free extracts, and purified enzymes.

The biotransformation processes are an amalgamation of various processes to carry out the process on an industrial scale, which needs the support of chemists, microbiologists, molecular biologists, and even engineers, making it

multidisciplinary. The whole cells consist the intact enzymatic machinery in their natural form and so is very stable and cost-effective to carry out transformation processes (Ishige et al. 2005) and hence is the matter of choice. This chapter focuses on the application of microorganisms and their enzymes in value addition through the process of biotransformation of various substrates. These biotransformation processes obey the principle of green chemistry generally and are known as white biotechnology.

13.2 History of Industrial Biotransformation

Microorganisms are known to have very serious implications in human life posing huge social and economic importance after the plants and animals. Without even being aware of it, man has been using microorganisms to produce foods and beverages. The commercial fermentation of alcohol, organic acids, and other chemicals started in the nineteenth century. Lactic acid was the first optically active product of fermentation. It is said that the history of vinegar production dates back around 2000 years BC, which is a microbial biotransformation process. Vinegar is one of the earliest and best-known examples of microbial oxidation produced via fermentation. The historic invention of the world's first practical microscope by Antony von Leeuwenhoek (1632–1723) prepared opened Pandora's box, and several scientific inventions came into existence. In 1858, Pasteur made an important contribution in the field of chemistry through his research on the optical isomerism of tartaric acid. He observed that incubation of the ammonium salt of racemic tartaric acid, using mold *Penicillium glaucum,* resulted in the formation of two different crystals of (+)- and (−)-tartaric acid. In 1897, Eduard Buchner (1897) proved that even in absence of live yeast cells, its cell-free extract could ferment carbohydrates to produce carbon dioxide and alcohol. This was a breakthrough observation leading to the use of inactive cells for biotransformations. Neuberg and Hirsch (1921) discovered that in the presence of yeast, the condensation of benzaldehyde with acetaldehyde formed optically active 1-hydroxy-1-phenyl-2-propanone. Peterson et al. (1952) reported that a fungus *Rhizopus arrhizus* selectively converts progesterone into 11*a*-hydroxyprogesterone, which is an intermediate in the synthesis of cortisone and hydrocortisone. In 1985, Anderson et al. reported the construction of a metabolically engineered strain that could synthesize 2-keto-l-gulonic acid, which is a key intermediate to produce l-ascorbic acid (vitamin C). Yamamoto et al. (1976) reported the continuous synthesis of L-malic acid from fumaric acid using immobilized *Brevibacterium ammoniagenes* as a whole-cell biocatalyst, while L- malic acid is an important compound used in the pharmaceutical industry. Kondo and Masuo (1961) developed a new process for the preparation of prednisolone from hydrocortisone using *Arthrobacter simplex* as a whole-cell biocatalyst. Some more industrially important products synthesized by using microorganisms as biocatalysts and its manufacturer are depicted in the Table 13.1.

Table 13.1 Selected products derived by biotransformation catalyzed by whole cells (De Carvalho 2017)

Product	Biocatalyst	Company
l-carnitine	*Agrobacterium* sp.	Lonza, Czech. Rep.
2-keto-l-gulonic acid	*Acetobacter* sp.	BASF, Merck, Cerester, Germany
Vitamin B_{12}	*P. denitrificans* *Propionibacterium shermanii*	Sanofi-Aventis, Hebei Huarong Pharmaceutical, NCPC victor and Hebei Yuxing bio-engineering
(R)-(−)-mandelic acid	Nitrilase	BASF and Mitsubishi rayon, Germany and Japan
Violacein (Swissaustral)	*Iodobacter* sp.	Swissaustral (Switzerland)
L-DOPA	*Erwinia herbicola*	Japanese academic groups and Ajinomoto (Japan)
L-lysine	*Corynebacterium glutamicum*	Ajinomoto (Japan)
L-glutamate	*Corynebacterium glutamicum*	Ajinomoto (Japan)

13.3 Biocatalyst-Mediated Green Chemistry

Industries and R & D laboratories working in the field of synthetic chemistry are having a social and regulatory pressure and hence are forced to adapt environment-friendly methods for the synthesis of various products. Anastas and Warner (1998) proposed the 12 principles of green chemistry, which are listed in Table 13.2. Biocatalysis offers certain benefits that allow the synthesis of products that are

Table 13.2 Biocatalysis obeying the principles of green chemistry (Sheldon and Woodley 2018)

Sr. no.	Green chemistry principle	Benefits offered by biocatalysis
1.	Waste prevention	Significantly reduce waste
2.	Atom economy	More atom- and step-economical
3.	Less hazardous synthesis of compounds	Generally low toxicity
4.	Design a chemical process for safe products	Not related to biocatalysis. Hence, no benefit offered
5.	Safe solvent and auxiliaries	Generally performed in water
6.	Energy efficiency	Mild and energy-efficient conditions required
7.	Renewable feedstocks	Enzymes are renewable
8.	Reduced derivatization	Avoids protection/deprotection steps
9.	Catalysis	Enzymes are catalysts
10.	Design for degradation	Not related to biocatalysis. Hence, no benefit offered
11.	Real-time analysis	Applicability to biocatalytic processes
12.	Inherently safer processes	Mild and safe conditions

very difficult or cannot be produced by conventional chemical synthesis methods. The high efficiency and specificity of chemical reactions could be achieved through the enzymes that could be naturally obtained from microorganisms, plants, and animals. The biocatalysts extend remarkable benefits of being resource-efficient, economical, energy-saving, and environment-friendly. Biocatalysis is being used for the synthesis of important chemicals of active pharmaceutical ingredients (API). The high selectivity is the most beneficial advantage of biocatalysts, which are often chiral (stereo), positional (regio), and functional group-specific (chemo) selectivity. Table 13.2 depicts the benefits of using enzymes and microorganisms. The most notable characteristics of biological catalysts include their inherent biodegradability and their ability to operate in an aqueous environment under normal environmental conditions.

As shown in Table 13.2., biocatalysis technology confirms ten out of twelve principles of green chemistry, while two principles do not support, because it relates to the product and design of the process. In a nutshell, it can be stated that biocatalysis is truly green and sustainable technology in the future.

The biocatalytic transformations could satisfy the above green chemistry principles, which confer privileges like a short span of reaction, fewer by-products, and economically more viable processes compared to traditional chemical synthesis methods. Biocatalysis is studied enthusiastically by researchers giving over 140 commercialized processes of industrial organic synthesis (Clouthier and Pelletier 2012; Huisman and Collier 2013; Summerton et al. 2016).

13.4 Advantage and Disadvantages of Whole-Cell Biocatalysis

Biocatalysis is lucrative for pharmaceutical industries, because it has the potential to provide safe and reliable industrial processes. Biocatalysts have a very large three-dimensional structure that makes multiple contact points with the substrate's remarkable selectivity (Truppo 2017). On the other hand, chemical synthesis processes do not carry environmentally benign reactions, while whole-cell biocatalysis offers numerous advantages and provides an effective and environmentally friendly substitute to produce bulk and fine chemicals (Straathof 2014). The benefits of biocatalysis over chemical catalysis (Zhao 2006; Lin and Tao 2017) are described below.

Advantages:

1. Generally, enzymes more efficient as its lower concentration is required to catalyze the chemical reaction.
2. Biocatalyst can be modified to increase selectivity, stability, and activity through modern molecular techniques.
3. Biocatalysts are selective to its substrate (including chemoselectivity, regioselectivity, diastereoselectivity, and enantioselectivity).
4. Biocatalysis reactions are performed under milder reaction conditions (typically in a pH range of 5–8, temperature range of 20–40 °C) and at an atmospheric pressure.

5. Multistep reactions are possible to be carried out by a single organism.
6. Recycling of biocatalyst is possible using the immobilization method.
7. Generally, they are environment-friendly.

Disadvantages:
1. Biocatalysts are susceptible to the substrate or product inhibition.
2. Generally, the biocatalytic reaction required aqueous medium.
3. In nature, enzymes are found in one enantiomeric form.
4. Limited operational range of conditions is available, since enzymes typically get denatured at high temperature, pH, and pressure.
5. Enzymes may cause allergic reactions to the human being.
6. There could be the generation of unwanted metabolic by-products that could be toxic to the cells and difficult to separate.
7. Reduced efficiency on recycling is generally observed during the reaction.

13.5 Biocatalytic Applications

Environmental regulation, competitiveness, and social responsibility pressurized the industries to develop more environmentally benign and economical processes for the manufacturing of industrially important compounds (Kirk et al. 2002). Biocatalysis has become an important tool in synthesizing fine chemicals, pharmaceuticals, agriculture-related products, etc. The scope of biocatalytic-mediated synthesis of industrially valuable compounds is rapidly expanding, owing to its ability to synthesize newer compounds. The identification and characterization of newer enzymes are increasing day by day because of the newly explored functions of enzymes and their use in the new chemical transformations that in majority follow the principles of green chemistry to ensuring it's the process is environment-friendly nature Biocatalytic applications are being increasingly adopted by several chemical industries to improve manufacturing, sustainability, and profitability, reducing the energy needs leading to the production of high-value chemicals. The acceptability of biotechnology-based methods on an industrial scale is increased due to the need for enantioselective compounds and fine chemicals, particularly in the pharma industry (Meyer and Turner 2009). Newer biocatalytic methods are being developed to cater to the need for specialty and bulk chemicals. Overall, application in the pharma sector heavily dominates.

Most of the biotransformation reactions that are carried out by microbes or enzymes involved oxidation, reduction, hydrolysis, condensation, isomerization, the C-C bonds formation, formation of a chiral molecule, hydroxylation, esterification, methylation, demethylation, isomerization, glycosylation, and hydrogenation, etc., while oxidation, reduction, isomerization, and hydrolysis reactions are the most common reactions observed in biotransformations. Biotransformation is presently being used to produce several chemicals.

13.5.1 Oxidation Reaction

Key oxidation steps carried out through microorganisms were employed in vitamin C production, (Reichstein and Grüssner 1934; Eggersdorfer et al. 2012) as well as for pharmaceutically important steroids (Horvath and Kramli 1947; Krámli and Horvath 1948; Wohlgemuth 2014). Oxidative catalysis is the main reaction in biocatalysis, which is environmentally benign. In view of green chemistry, the whole cells or enzyme-catalyzed oxidation process offers selectivity, which is the biggest lacuna of chemocatalysts (Hollmann et al. 2011). The oxidative biocatalysts (oxygenases, peroxidases, oxidases, and dehydrogenases) play an important role in these oxidative processes. The oxidative biocatalyst displays high redox potentials, which enable them to facilitate reaction with chemically resistant (stable) starting compounds. Several examples of oxidations of primary alcohols to the corresponding acids were reported over the years. Regioselectivity is very important in the case of the oxidation of carbohydrates. In the Reichstein process, *Gluconobacter oxydans* is used as a catalyst that selectively catalyzes the oxidation of sorbitol to ascorbic acid (Fig. 13.1a) (Hancock and Viola 2002).

Gluconobacter oxydans is also used for selective oxidation of glycerol (Bauer et al. 2005), ribitol (De Muynck et al. 2006), and *N*-butylglucamine (Landis et al. 2002).

Recently, Cang et al. (2019) reported 5-hydroxymethylfurfural (HMF) to produce useful derivative 5-hydroxymethylfuroic acid (HMFCA) in the presence of *Deinococcus wulumuqiensis* R12, a new robust whole-cell biocatalyst for the selective oxidation reaction. The selective oxidation of other structurally diverse aldehydes to their corresponding acids was catalyzed by the whole cells of *D. wulumuqiensis* R12 (Fig. 13.1.b) with a good yield and high selectivity, indicating broad substrate scope. The authors compared the resting as well as growing cells to catalyze the oxidation of 5-hydroxymethylfurfural (HMF), where the resting cells showed an excellent catalytic performance over a broad range of pH and temperature and tolerance to a high concentration of substrate HMF and the product HMFCA. A high yield of HMFCA (~90%) was achieved at 300 mM substrate concentration.

Then, Chen et al. 2014, reported the selective oxidation of sulfides to optically active sulfoxides using *Pseudomonas monteilii* CCTCC M2013683. This strain exhibited high activity, substrate tolerance, and stereoselectivity to perform oxidation of organic sulfides. The yields of various chiral sulfoxides were 54–99% with 63–99% enantiomeric excess (e.e) (Fig. 13.1c).

Various aromatic aldehydes, such as 1- and 2-naphthaldehydes, cinnamaldehyde, vanillin, syringaldehyde, and benzaldehydes, have been converted to their corresponding carboxylic acids by the *Sphingomonas* sp. aldehyde dehydrogenase PhnN, which was overexpressed in *E. coli*. The recombinant *E. coli* cells converted 1,4-dihydroxymethylnaphthalene to 1-hydroxymethyl-4-naphthoic acid (Peng et al. 2005). In 2020, Hoshino and colleagues discovered the new oxidation process for the synthesis of both aromatic and aliphatic carboxylic acids using a *Geotrichum candidum* aldehyde dehydrogenase (GcALDH) enzyme (Hoshino et al. 2020). The

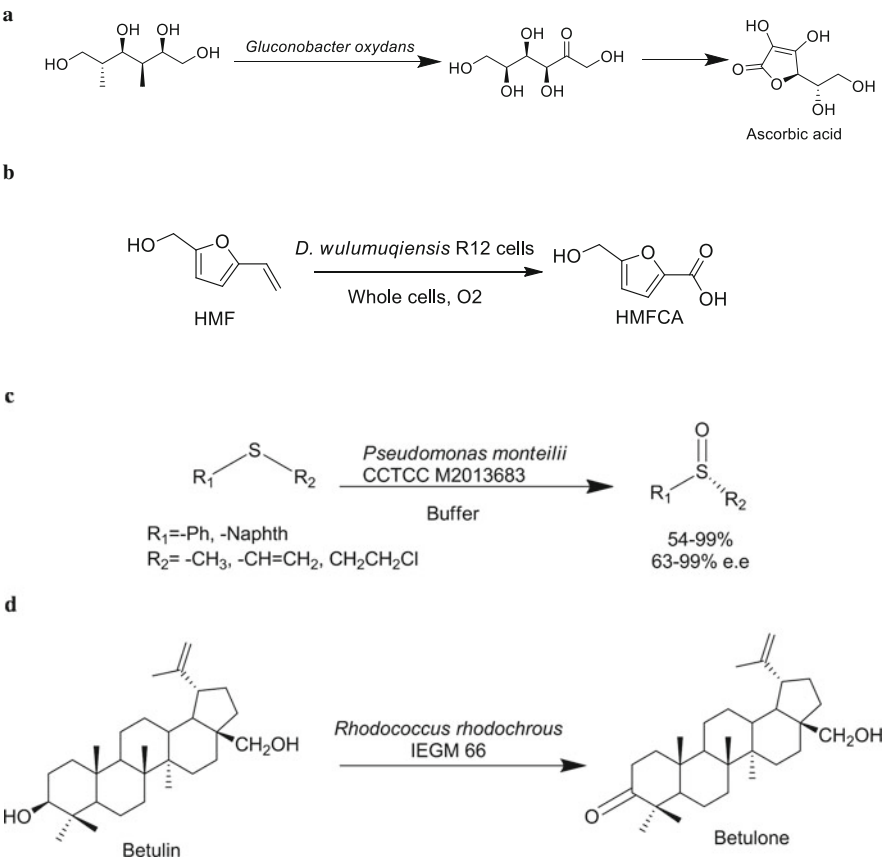

Fig. 13.1 Oxidation reactions catalyzed by microbes – (**a**): sorbitol to ascorbic acid selectively catalyzed by *Gluconobacter oxydans*, a key step of the Reichstein process; (**b**): chemoselective transformation of 5-hydroxymethylfurfural (HMF) to 5-hydroxymethylfuroic acid (HMFCA) with whole *D. wulumuqiensis* R12 cells; (**c**): whole-cell biocatalyst *Pseudomonas monteilii* catalyzed to give the enantioselective sulfoxides; (**d**): regioselective transformation of betulin to betulone using *Rhodococcus rhodochrous* IEGM 66

author found that the enzyme overexpressed in *E. coli* can catalyze the oxidation of various aromatic and aliphatic aldehydes. The preparative-scale production of oxidation of aldehydes under mild conditions using a green catalyst, GcALDH, was successfully demonstrated.

Kiebist et al. 2017 reported the modification of testosterone through an enzyme peroxygenase named *Cgl*UPO from a fungus *Chaetomium globosum* that facilitated the direct incorporation of epoxy and hydroxy functionalities into that molecule along with hydrogen peroxidase as a co-substrate bringing two oxygenated products: the 4,5-epoxide of testosterone in β-configuration and 16α-hydroxytestosterone. This reaction was performed on a smaller scale of 100 mg level yielding ~90% of the epoxide and 10% of the hydroxylated product with 96% purity of each, while this

reaction is very useful for the synthesis of pharmaceutically relevant steroidal molecules. Pyo et al. 2012, synthesized methacrylic acid from 2-methyl-1,3-propanediol (3H2MPA) using alcohol dehydrogenase and aldehyde dehydrogenase of *Gluconobacter oxydans* with a yield of 95–100% with over 95% selectivity to 3H2MPA. Further, the final product of the bio-oxidation 3H2MPA was converted to methacrylic acid, using chemical catalyst titanium dioxide. This process provided a new tool to produce methacrylic acid.

Chaudhari et al. (2010) described the process of cholesterol biotransformation using the whole cell of *Chryseobacterium gleum*. These bacteria, through their cholesterol oxidase enzyme, transformed cholesterol to androsta1,4-diene-3,17-dione (ADD) efficiently. The elucidation of the probable pathway of formation of ADD from cholesterol by *C. gleum* was described. In this process, 0.076 g ADD was produced from 1 g cholesterol per liter. In the similar way, generally recognized as safe probiotic bacterium, *Lactobacillus helveticus* has also been reported to transform 1 g cholesterol to 0.05 g of androsta-1,4-diene-3,17-dione, and 0.04 g of androst4-ene-3,17-dione within 48 h at 37 °C (Ahire et al. 2012).

Betulin, a key compound of birch bark extract, a pentacyclic triterpenoid, occurs widely in numerous plants (*Betula* spp.). Betulin has attracted more attention because it has pharmacological activities (Alakurtti et al. 2006). In 2012, Mao and coworkers tested 47 microbial strains for the transformation of botulin to betulone (Mao et al. 2012). The highest botulin-transforming activity was observed strain as *Rhodotorula mucilaginosa* with 52.65% yield was obtained. In 2003, Grishko and colleagues reported the biotransformation of regioselective oxidation of a 3-hydroxyl group of botulin to betulone using *Rhodococcus rhodochrous* IEGM 66. Out of 55 strains of *Rhodococcus* were tested for this transformation. The *Rhodococcus rhodochrous* IEGM 66 showed very high betulin-transforming ability with 75% betulone yield (Fig. 13.1d).

Chiral compound 1-phenyl-1,2-ethanediol (PED) is an important feedstuff for the preparation of pharmaceuticals, agrochemicals, and cosmetics. Peng et al. (2019) reported the whole-cell biocatalyst *Kurthia gibsonii* SC0312 efficiently and selectively oxidize racemic (R) 1-phenyl-1,2-ethanediol to (S)-PED in an aqueous reaction system at pH 5.5–8.5, 25–45 °C with 41% yield, and 94% optical purity. Peng et al. (2020) developed a new biphasic system for the asymmetric resolution of racemic 1-phenyl-1,2-ethanediol (PED) to (*S*)-PED by *Kurthia gibsonii* SC0312. The advantage of this system was that oxidized product has been efficiently extracted by dibutyl phthalate, with yields of 47.7% and over 99% optical purity of (S)-PED.

13.5.2 Hydroxylation Reaction

In living organisms, hydroxylation is the most common reaction carried out, where a carbon-hydrogen is converted to a carbon-hydroxyl bond. Several industrially important chemicals are being produced by hydroxylation reaction and are also used in bioremediation (Holland and Weber 2000). In 1952, the first commercially

employed bioprocess involved the *Rhizopus nigricans* as whole-cell biocatalyst-mediated transformation of progesterone to 11 α-hydroxyprogesterone (Fig. 13.2a).

A mutant strain of *Aspergillus ochraceus* converted progesterone to 11α-hydroxy progesterone with 90% (Somal and Chopra 1985). In 1988, Liu et al. reported the stereoselective hydroxylation of 1,4-cineole catalyzed by growing whole-cell biocatalysts: *Bacillus cereus* and *Streptomyces griseus*. The biocatalysts introduced the hydroxyl groups at the 2- and 8-positions of the substrate of monoterpene, bringing two pairs of endo and exo enantiomers.

The compactin is a cholesterol-lowering compound. It inhibits the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase, which is involved in cholesterol biosynthesis. The C6 hydroxylated compactin, called pravastatin, can reduce the bad cholesterol and fats, which has the potential to lower the risk of cardiovascular disease. Hence, many researchers explored this biotransformation reaction for the conversion of compactin to pravastatin. In 1997, Peng et al. reported the conversion of compactin to pravastatin using *Actinomadura* sp. (Peng et al. 1997) (Fig. 13.2b), while *Streptomyces* sp. was also reported to produce 340 mg l^{-1} of pravastatin from 750 mg compactin l^{-1} in 24 h (Park et al. 2003). Recently, Yao et al. (2017) demonstrated that CYP105D7 of *Streptomyces avermitilis* catalyzed the hydroxylation of compactin to obtain pravastatin (Fig. 13.2b).

Chemical methods have limitations to bring regiospecific hydroxylation of aromatic compounds. Fwjmm et al. (1990) produced 6-hydroxynicotinic acid from nicotinic acid by microorganisms like *Pseudomonas acidovorans* and also by *Achromobacter xylosoxidans* (Fig. 13.2c). Prenalterol [1-(4-hydroxyphenoxy)-3-isopropylamino-propane-2-ol], which acts as cardiac stimulant, could be synthesized by regioselective para-hydroxylation of isopropyl-amino-3 phenoxypropane-2-ol by *Cunninghamella echinulata* with 0.85% yield (Pasutto et al. 1987).

Cytochrome P450 monooxygenase systems play an important role in the metabolism of a variety of hydrophobic compounds. They are involved in the modification of steroids, fatty acids, vitamins, and other biological processes. Bleif et al. (2012) developed the CYP106A2 dependent recombinant expression system in *B. megaterium* which was successfully used for the hydroxylation of pentacyclic triterpene 11-keto-boswellic acid to 15α-hydroxy-keto-boswellic acid using whole cells while the product selectivity 80 % was achieved (Fig. 13.2d).

Hydroxylated products of daidzein and genistein are pharmaceutically important compounds. Such products have antioxidant properties with anticarcinogenic, cholesterol-lowering, and cardiovascular protective effects (Klus and Barz 1995; Esaki et al. 1999; Rüfer and Kulling 2006).

Roh et al. (2009a), reported the regioselective hydroxylation of daidzein and genistein using whole cells of *Streptomyces avermitilis* that showed high activity to produce 3,4,7-trihydroxyisoflavone and 3,4,5,7-tetrahydroxyisoflavone (Fig. 13.2e). Roh et al. (2009a, b) further described the hydroxylation of daidzein, yielding 7,3,4-trihydroxyisoflavone using a recombinant whole-cell system. The cytochrome CYP107H1, from *Bacillus subtilis* 168, and redox partner putidaredoxin reductase (*camA*) and putidaredoxin (*camB*), from *Pseudomonas putida*, were transformed

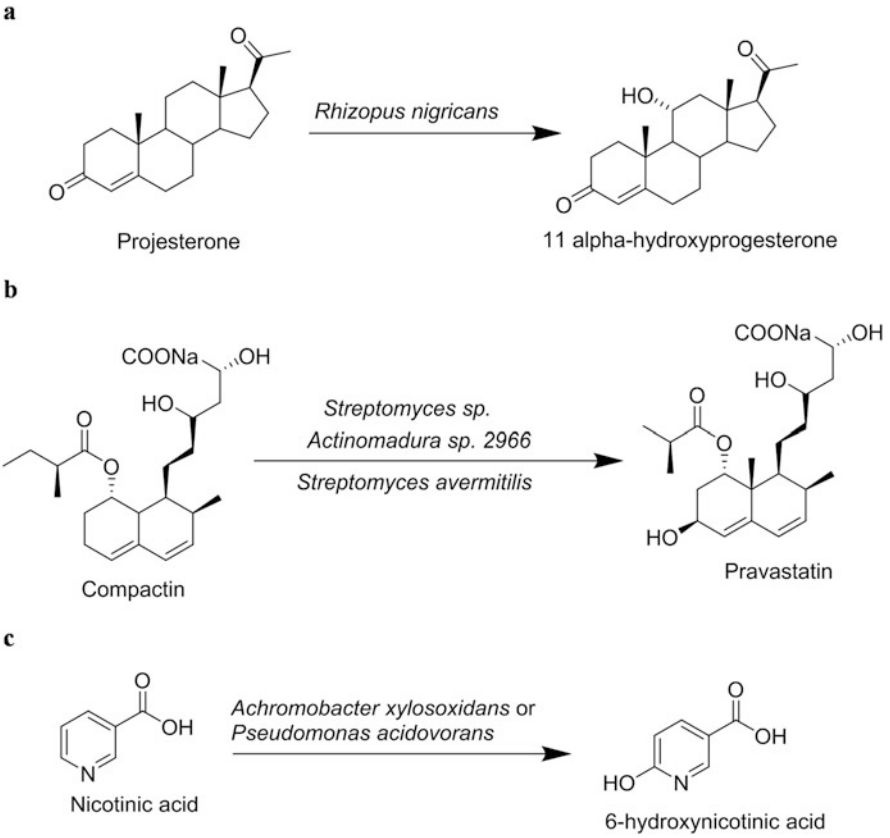

Fig. 13.2 Hydroxylation reactions catalyzed by various microorganisms – (**a**): Biotransformation of progesterone to 11α-hydroxyprogesterone by fungus *Rhizopus nigricans*. (**b**): Bioconversion of compactin to pravastatin using biocatalysts; (**c**): regioselective catalysis of aromatics. (**d**): CYP106A2-catalysis of KBA at the 15th position. (**e**): Conversion of daidzein and genistein to 3′,4′,7-trihydroxyisoflavone and 3′,4′,5,7-tetrahydroxyisoflavone using *Streptomyces avermitilis* MA-4680. (**f**): Daidzein catalyzed by CYP107H1 with redox partner. (**g**): Conversion of DHEA by *B. bassiana*. (**h**): Biotransformation of (R)-2-phenoxypropionic acid (POPS) to (R)-2-(4-hydroxyphenoxy) propionic acid (HPOPS). (**i**): Conversion of 2-phenoxypropionic acid (POPA) to 2-(4-hydroxyphenoxy) propionic acid (HPOPA)

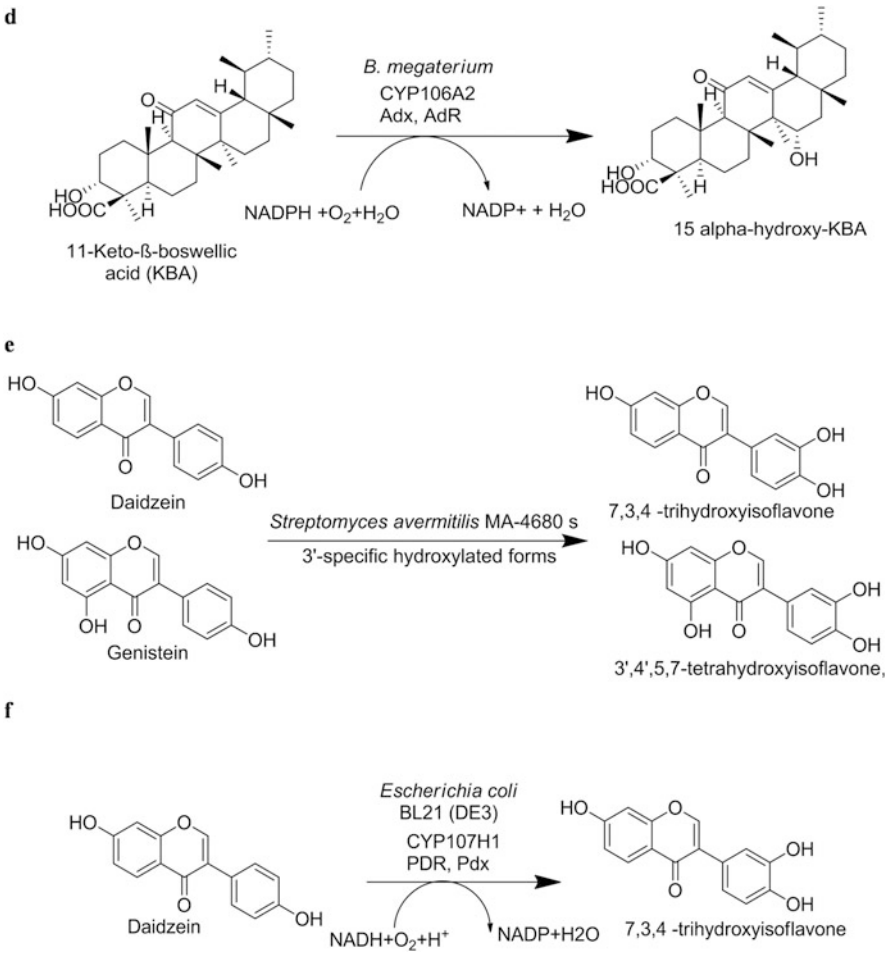

Fig. 13.2 (continued)

into a culture of *Escherichia coli* BL21 (DE3), which was further employed for 3-*ortho*-specific hydroxylation activity of daidzein (Fig. 13.2f).

In skin cells, prohormone vitamin D_3 was synthesized from 7-dehydrocholesterol under UV radiation (Tuckey et al. 2018), or it can even be acquired from the diet (Carlberg and Haq 2018). Recently, Tang et al. (2020) developed an alternative bioprocess for the production of 25-hydroxyvitamin D3. This efficient protocol was catalyzed by *Bacillus cereus* of soil habitat using vitamin D3 as a sole carbon source, where under optimized conditions, a 796 mg/L of 25-hydroxyvitamin D3 was achieved after 48 h at the shake flask level and later in a 5 L fermenter with a yield of 41.5%.

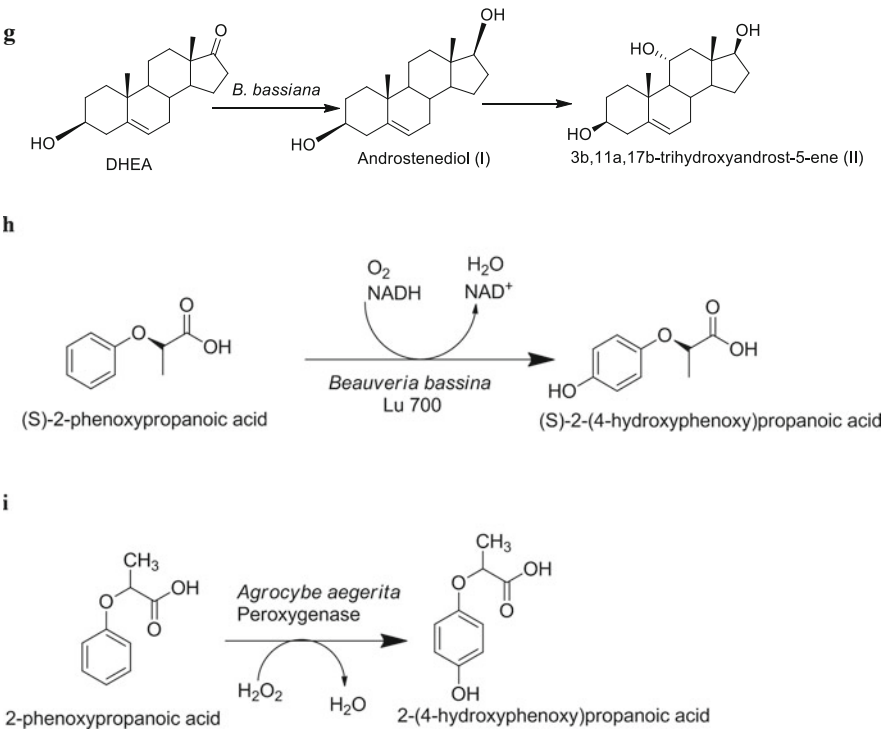

Fig. 13.2 (continued)

Dehydroepiandrosterone (DHEA) is a steroid hormone in humans that is a precursor to other hormones, like androstenedione, testosterone, estrone, and estradiol. Gonzalez et al. 2017, discovered the eco-friendly process for the synthesis of valuable 11a-hydroxy steroids. The *B. bassiana* transformed 3- hydroxy-17-oxo DHEA to the 3,17-hydroxysteroid (I), which then underwent a 11α-hydroxylation to form 3,11,17-hydroxysteroid (II), where no other by-products were formed. In this work, the resting cells of fugus were used where 63% of the substrate was converted to products, while in the case of growing cells only 38% of substrate conversion took place. The higher amounts of steroids I and II were synthesized using resting cells (Fig. 13.2g).

Holland and Lakshmaiah (1999), investigated the microbial biotransformation of 2-oxatestosterone to hydroxylate conventional steroids using various fungi and bacteria. *A. ochraceus* and *B. megaterium* gave products of 11α- and 15-β-hydroxylation, respectively, while *C. lunata* gave C-11β- and C-14α-hydroxylated products, and *R. arrhizus* produced only the 6β-hydroxy derivative.

Yildirim and Kuru (2017) studied the hydroxylation of epiandrosterone using *Aspergillus candidus* MRC 22634, obtaining 10-hydroxylated metabolites.

A. candidus hydroxylate 1 predominantly at C-11a, C-1a, and C-15b with minor hydroxylations occurring at C-14a and C-7a. Through this study, two of the metabolites, 1a,3a-dihydroxy-5a-androstan-17-one 4 and 15b,17b-dihydroxy-5a-androstan-3-one 7, were identified as new compounds.

The production of (R)-2-(4-hydroxyphenoxy) propionic acid (HPOPS) is a valuable intermediate for the synthesis of enantiopure aryloxyphenoxypropionic acid-type herbicides. The hydroxylation of (R)-2-phenoxypropionic acid (POPS) to (R)-2-(4-hydroxyphenoxy) propionic acid (HPOPS) was carried out by *Beauveria bassiana* and its mutant. This strain was catalyzing the selective monohydroxylation of other tested aromatic carboxylic acids (Dingler et al. 1996) (Fig. 13.2h).

Kinne et al. (2008) reported that the extracellular heme-thiolate peroxygenase from mushroom *Agrocybe aegerita* catalyzed the H_2O_2-dependent hydroxylation of 2-phenoxypropionic acid (POPA) to give the herbicide precursor 2-(4-hydroxyphenoxy) propionic acid (HPOPA), having an isomeric purity near 98% and yields of desired R-isomer of HPOPA with an enantiomeric excess of 60% (Fig. 13.2i).

Apart from the above-stated reactions, there are several hydroxylation reactions reported in the literature, which are carried out by many different microorganisms as the catalyst.

13.5.3 Reduction Reaction

The use of whole cells offers several advantages in comparison with isolated enzymes, where the most common advantage is the use of mild conditions with high chemo-, regio-, and stereoselectivities. The chemical methods for reducing carbon-carbon double bonds demand harsh environmental conditions and are non-selective in nature. The biological reduction of C=C double bonds is in high demand due to its advantages.

Ferreira et al. (2014) described immobilized *Penicillium citrinum* on various matrices and its whole cells to catalyze the reduction of chalcones to hydrochalcones by enoate reductases with good to excellent yields in both cases (Fig. 13.3a).

The microbial biotransformation of widely available monoterpenoids, such as carvone, menthol, citronellol, and geraniol derivatives, added value to make feedstuff for perfume, food, and pharmaceutical industries. Silva et al. (2012) reported the asymmetric reduction of (4R)-(−)-carvone catalyzed by baker's yeast *Saccharomyces cerevisiae* in aqueous mono- and biphasic systems (Fig. 13.3b). In this process, the concentration of the yeast, substrate, temperature, and pH was evaluated. Carvone can be used as an intermediate for the synthesis of some molecules of biological interest, such as (−)-thujopsene (Zhang et al. 2008), 10-acetoxy1H,7H-guaia-4,11-dien-3-one (Blay et al. 2005), and (+)-decipienin (Macías et al. 2000).

Müller et al. (2006) reported abilities of abacterial strain *Zymomonas mobilis* and a fungal strain *Candida rugosa* to reduce citral to citronellal with high enantioselectivity (Fig. 13.3c). The bacterial strain produced preferentially the (S)-

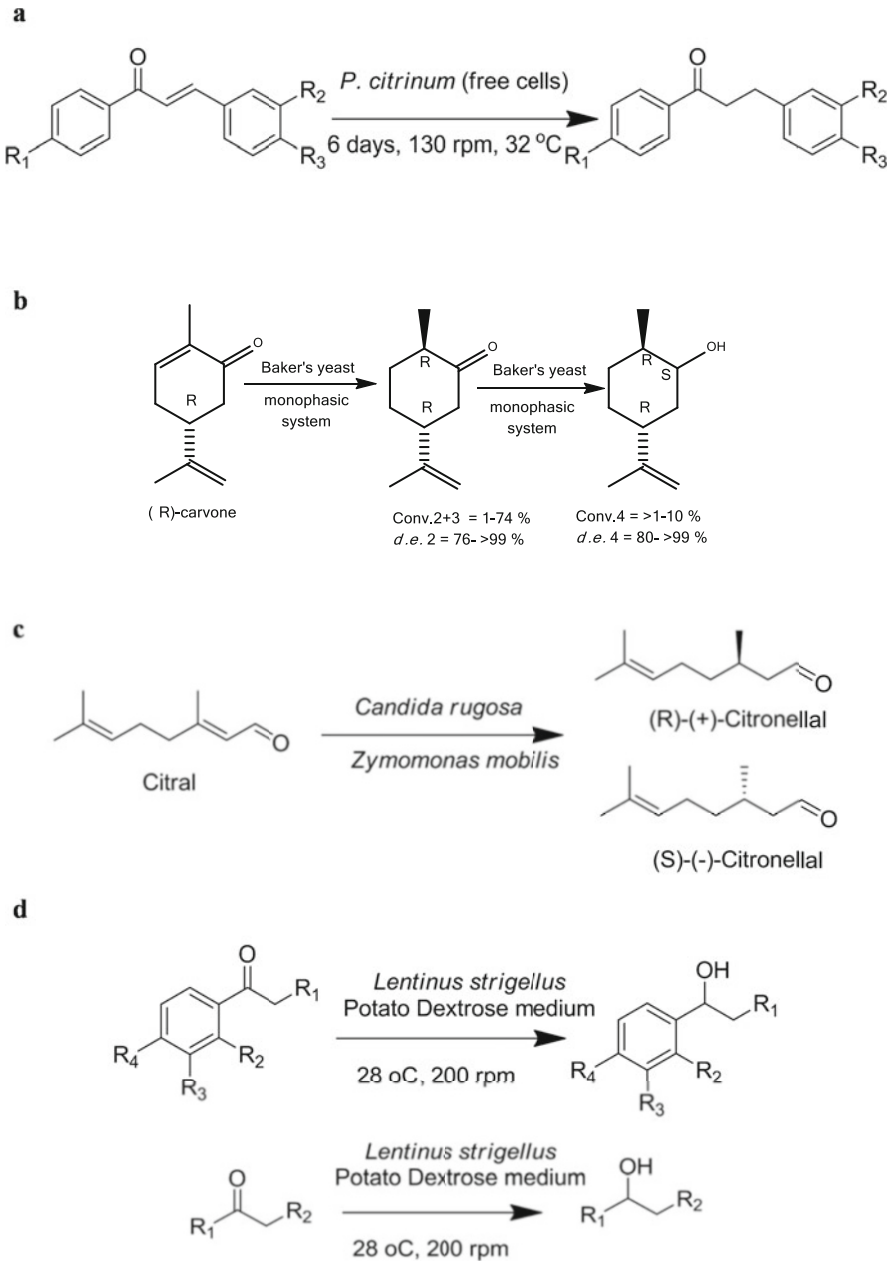

Fig. 13.3 Reduction reaction catalyzed by various microorganisms. (**a**): Chalcones to hydrochalcones catalyzed by whole cells of *P. citrinum* CBMAI 1186. (**b**): Biotransformation of (R)-carvone catalyzed by Baker's yeast. (**c**): Citral (geranial and neral) into citronellal by yeast *Candida rugosa* and bacterium *Zymomonas mobilis*. (**d**): Bioreduction of prochiral aromatic acetophenone and aliphatic ketones using *Lentinus strigellus*. (**e**): 2-Methyl-1-phenylpropan-1-one to (R)-2-methyl-1-phenylpropan-1-ol using *Lactobacillus paracasei* BD101. (**f**): 6-Chlorochroman-4-one to (S)-6-chlorochroman-4-ol using whole cells of *L. paracasei* BD87E6.

enantiomer of citronellal with e.e. values of >99%, while *Candida rugosa* produced predominantly (R)-citronellal with 98% enantioselectivity. Rather than an aqueous system, a mixture of aqueous/organic systems was better in identifying microbes for this transformation.

The chiral alcohols have several applications as bioactive compounds and adequate precursors for the synthesis of various compounds (Breuer et al. 2004). The asymmetric reduction of prochiral ketones is used to produce enantiomerically pure alcohols.

Barros-Filho et al. (2009) reported the reduction of prochiral aromatic and aliphatic ketones to the corresponding enantiomerically enriched alcohols by using *Letinus strigellus* in moderate to excellent conversions and enantioselectivities (Fig. 13.3d).

The chiral carbinols are useful precursors in the synthesis of various drugs. The biocatalyzed methods are generally used on an industrial scale via the asymmetric bioreduction of cheap prochiral ketones and the resolution of racemates.

Şahin (2020a) reported the enantioselective bioreduction of 2-methyl-1-phenylpropan-1-one ketone to (R)-2-methyl-1-phenylpropan-1-ol ((R)-2) catalyzed by *Lactobacillus paracasei* as a whole-cell biocatalyst (Fig. 13.3e).

Şahin (2020b) reported *L. paracasei* as a whole-cell biocatalyst to carry out the asymmetric reduction of prochiral aromatic and heteroaromatic ketones and fused bicyclic ketone into chiral carbinols with excellent enantiomeric purity >99%. This study established the production of enatiopure (S-6-chlorochroman-4-ol) by the whole-cell biocatalyst (Fig. 13.3f).

Baydaş et al. (2020a) reported the *Lactobacillus kefiri* P2 as a biocatalyst for the asymmetric reduction of prochiral ketone acetophenone, bringing secondary chiral aromatic alcohol with 99% enantiomeric excess (Fig. 13.3g).

Recently, Baydaş et al. (2020b) reported the efficient method using whole-cell *Lactobacillus kefiri*-mediated enantioselective reduction of various prochiral ketones. The study indicates selective bioreduction of different ketones to their respective (R)-alcohols in low to high selectivity (0- > 99%) with good yields (11–96%). The gram-scale production was carried out, and 9.70 g of (R)-2-

Fig. 13.3 (continued) (**g**): Reduction of acetophenones to chiral alcohol by *Lactobacillus kefiri*. (**h**): Bioreduction of various prochiral ketones using *Lactobacillus kefiri* P2. (**i**): Biotransformation of HMF to BHMF in the buffer using *Meyerozyma guilliermondii SC1103*. (**j**): -5-Hydroxymethylfurfural (HMF) to 2, 5-bis hydroxymethyl HMF (BHMF) using the whole cells of *Aureobasidium subglaciale* F134. (**k**): 3-Chloro-1-(2-thienyl)propanone to (S)-3-Chloro-1-(2-thienyl)-1-propanol by *Candida pseudotropicalis* 104. (**l**): Chemoenzymatic synthesis of (S)-rivastigmine in four steps using a biocatalyst *Lactobacillus reuteri*. (**m**): Bioreduction of PTX to LSF by *Lactobacillus kefiri*

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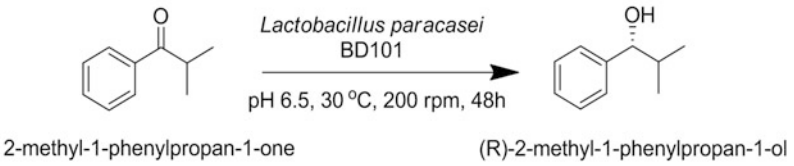

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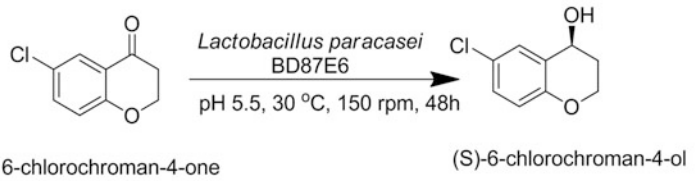

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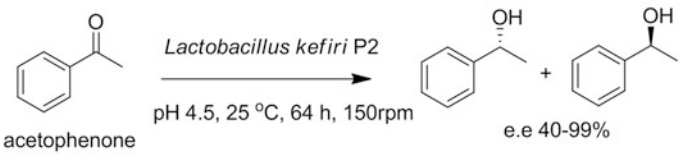

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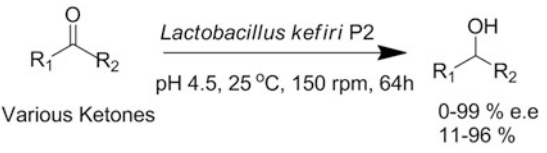

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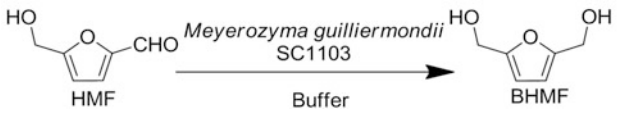

Fig. 13.3 (continued)

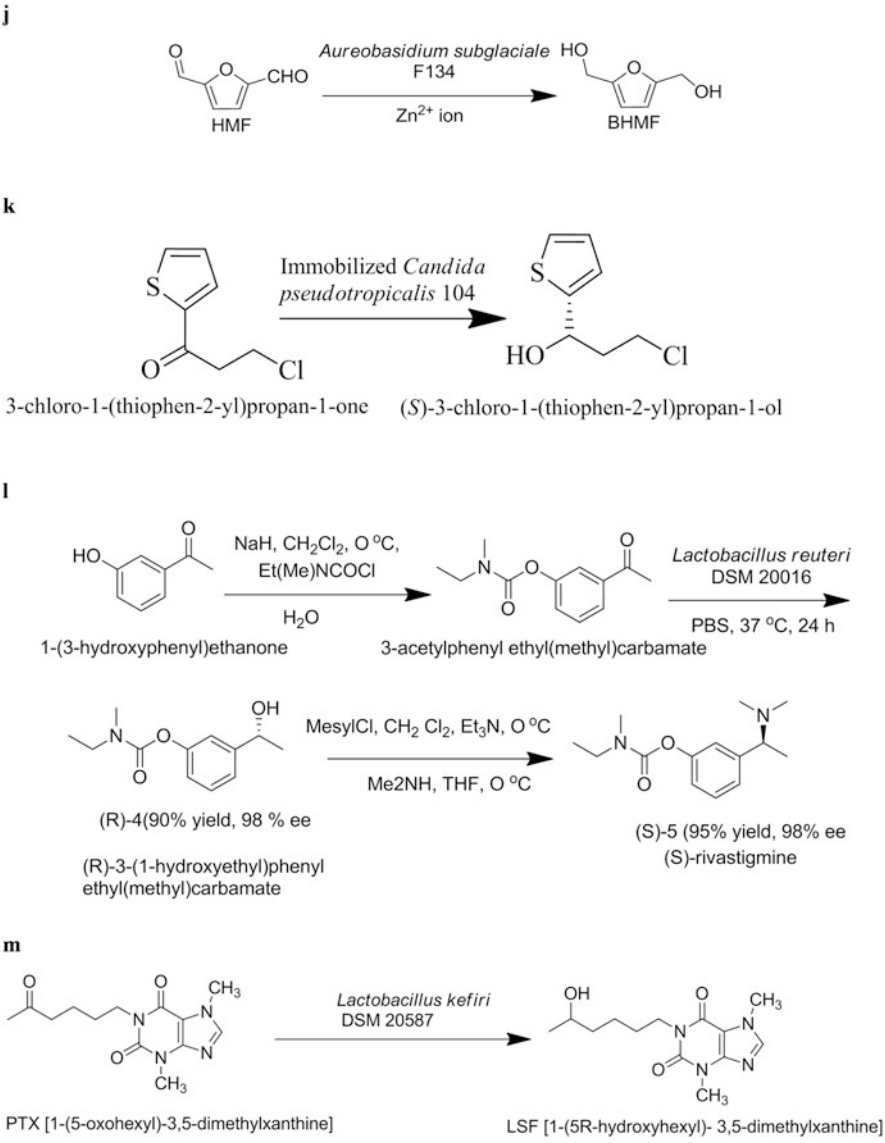

Fig. 13.3 (continued)

methyl-1-phenylpropan-1-ol in enantiomerically pure form was obtained in 96% yield (Fig. 13.3h).

In recent years, the 5-hydroxymethylfurfural (HMF) is a very important compound derived from renewable biomass. The functionalized molecule with an aldehyde group, a hydroxyl group, and a furan ring provides great potential to produce a wide variety of valuable chemicals. From the various valuable compounds

derived from biomass, HMF is identified to be a top building block chemical (Biddy et al. 2016). HMF can be further transformed into value-added chemicals, including 2,5-furandicarboxylic acid (Neațu et al. 2016; Siyo et al. 2014), 2,5-dimethyl furan (Li et al. 2017a, b; Nishimura et al. 2014), and levulinic acid (Zhang et al. 2016), and other chemicals through oxidation, hydrogenation, hydrolysis, etc.

Li et al. (2017a, b) developed a new highly HMF-tolerant yeast strain *Meyerozyma guilliermondii* to use in the biocatalytic reduction of HMF to 2,5-bis (hydroxymethyl)furan (BHMF). A high BHMF yield of 86% with excellent selectivity of >99% was afforded by using 100 mM HMF as a substrate within 12 h in the presence of 100 mM glucose as a co-substrate (Fig.13.3i).

Recently, Chen et al. (2021) reported an efficient bioreduction process of biomass-derived 5-hydroxymethylfurfural (HMF) to 2,5-bis(hydroxymethyl)furan (BHMF) by using yeast-like fungus *Aureobasidium subglaciale* as a whole-cell biocatalyst. The cells of *Aureobasidium subglaciale* F134 exhibited high tolerance and excellent selectivity to HMF in a broad pH range, while the catalytic efficiency was enhanced by Zn^{2+} ions. Under the controlled conditions, 180 mM HMF substrate was converted into BHMF with a yield of 82% and 97% selectivity in 9 h (Fig. 13.3j).

Enantio- and diastereomerically pure diols are important for the synthesis of pharmaceuticals, agrochemicals, and fine chemicals. In this view, Haberland et al. (2002a, b) developed a continuous production process for optically active pure (2R,5R)-hexanediol via reduction by whole cells of *Lactobacillus kefir* in a 2-L continuously operated membrane reactor. The enantioselectivity and diastereoselectivity were more than 99% were obtained. The yield for the continuous process over 5 days was 64 g,L^{-1} ,d^{-1}. At the same time, the same author conducted a similar experiment which was carried out using *Lactobacillus kefir* DSM as a biocatalyst for the reduction of (2R,5R)-hexanediol starting from (2,5)-hexanedione in fed-batch experiments was demonstrated. The quantitative yields with enantiomeric excess >99% and diastereomeric excess >99% were observed.

The (S)-3-chloro-1-(2-thienyl)-1-propanol is a key intermediate for the synthesis of (S)-duloxetine obtained by the reduction of 3-chloro-1-(2-thienyl)propanone, using the chemical and biotransformation method. Duloxetine is a medication used to treat major depressive disorder, generalized anxiety, fibromyalgia, and neuropathic pain. Zhimin et al. (2012) reported the reduction of 3-chloro-1-(2-thienyl) propanone to the corresponding (S)-3-chloro-1-(2-thienyl)-1-propanol using liquid-core immobilized *Candida pseudotropicalis* 104, where liquid-core immobilized cells were reused 11 times. In this work, the continuous reduction of 3-chloro-1-(2-thienyl)propanone in the membrane reactor with liquid-core immobilized cells as a catalyst. Conversion and enantiomeric excess of (S)-3-chloro-1-(2-thienyl)-1-propanol reached 100% and > 99% in a continuous reduction of 12 g/L 3-chloro-1-(2-thienyl)propanone for 10 days (Fig. 13.3k).

The total synthesis of an important API (active pharmaceutical ingredient) is a key challenge in the biotransformation field. The (S)-rivastigmine is very useful in the treatment of mild to moderate dementia in patients with Alzheimer's disease and Parkinson's disease because of inhibition of acetylcholinesterase and butyrylcholinesterase (Kandiah et al. 2017; Bhanja and Jena 2012). Vitale et al.

(2013) reported a simple chemoenzymatic process to produce (S)-rivastigmine (Fig. 13.3l). The total synthesis of (S)-rivastigmine was then performed in four steps. The reduction of 3-acetylphenyl-N-ethyl-N-methylcarbamate by whole cells of *Lactobacillus reuteri* to the corresponding secondary alcohol (R)-3(1 hydroxyethyl)phenylethyl(methyl)carbamate under mild conditions. The latter is finally converted into the target drug (S)-5 rivastigmine (Han et al. 2010; Yan et al. 2013).

Shang et al. 2017, reported the synthesis of (R)-2-chloro-1-(2,4-dichlorophenyl) ethanol using ketoreductase from *Scheffersomyces stipitis* CBS 6045. The 2-chloro-1-(2,4-dichlorophenyl)ethanone is an intermediate in the synthesis of common antifungal agents.

Scully et al. (2019), investigated the whole-cell biocatalyst *Thermoanaerobacter pseudoethanolicus* (DSM 2355) reduces the C1-C8 short-chain fatty acids (SCFAs) to corresponding alcohols using glucose as a carbon source with yields between 21.0 and 61.0%.

Zadlo et al. (2016) reported the enantioselective reduction of ethyl 3-oxo-5-phenylpentanoate using the various microorganisms whole-cell-mediated reduction of ethyl 3-oxo-5-phenylpentanoate. Cofactors, recycling systems, and 2-propanol amounts were optimized for selected biocatalysts, leading to excellent enantiomeric excesses for the obtained hydroxy ester with up to 99% e.e. Out of the tested biocatalysts, isolate *Gordonia* sp. NAMBN063A and *Paracoccus pantotrophus* afforded (*S*)-configured product **1** with enantiomeric excesses of 77 and 92%, respectively. Moreover, the use of *Arthrobacter* sp., isolates *Actinomyces* sp. SRBAN053, isolate ARG-AN024, or *Sphingomonas* sp. HXN200 provided the opposite (*R*) enantiomer with excellent enantiomeric excesses of up to 98%. Mitsukura et al. (2010) reported the reduction of 2-methyl-1-pyrroline by whole cells of *Streptomyces* species GF3587 and 3546, which obtained highly enantioselective 2-methyl-1-pyrroline (2-MPN). In this process, whole-cell catalyst produced R-2-methylpyrrolidine (*R*-2-MP) with 99.2% e.e. and 27.5 mM S-2-MP (92.3% e.e.) from 2-MPN at 91–92% conversion in the presence of glucose.

Lisofylline (LSF) is a drug molecule with anti-inflammatory activities which could prevent type 1 diabetes in preclinical models. Pękala et al. (2007) reported the reduction of pentoxifylline [1-(5-oxohexyl)-3,5-dimethylxanthine] to LSF [1-(5R-hydroxyhexyl)-3,5-dimethylxanthine] using whole-cell *Lactobacillus kefiri* DSM 20587 with high enantiopurity (98% e.e) and a very good yield (100%) (Fig.13.3m).

13.5.4 Hydrolysis Reaction

The enzyme-catalyzed reactions and hydrolytic transformations, including amide and ester bonds, are comparatively easy using proteases, esterases, or lipases. About 50% of research in the field of biotransformation has been performed using the hydrolase class of enzymes. Only hydrolases class has technological significance, since these enzymes are well understood and perform a wide range of reactions,

being extracellular, robust, and commercially available, not requiring coenzymes or cofactors. The enzymes belonging to hydrolase class of enzymes are generally used in the hydrolysis of various bonds and therefore have the potential to catalyze many biotransformation reactions.

Botes et al. (1998), reported the hydrolysis of 1,2-epoxyoctane, using the epoxide hydrolase enzyme from the various yeast strain. All these strains preferentially hydrolyzed (R)-1,2-epoxyoctane to (R)-1,2-octanediol with an excellent enantioselectivity (E > 200) for 1,2-epopxyoctane which is reported for the first time.

Thakur et al. (2018) reported the synthesis of 4-hydroxyphenylacetic acid (4-HPAA) from 4-hydroxyphenylacetonitrile (4-HPAN) using whole-cell arylacetonitrilase of *Alcaligenes faecalis*. *A. faecalis* nitrilase preferably hydrolyzed 4-HPAN, 4-aminoyphenylacetonitrile, mandelonitrile, and phenylacetonitrile to their corresponding acids (Fig. 13.4a).

The 5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1H-indene-2-carboxylic acid methyl ester is an intermediate for the synthesis of indoxacarb. Indoxacarb is an oxadiazine pesticide against lepidopteran larvae. The S-enantiomer of indoxacarb is active against the insects, while the R-enantiomer was inactive (Wing et al. 1998), and hence, the synthesis of (S)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1H-indene-2-carboxylic acid methyl ester was of high importance as the pesticide. In this regard, Zhang et al. (2019) explored *B. cereus* to catalyze a new biocatalytic route for preparing the indoxacarb chiral intermediates (*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester. The *Bacillus cereus* exhibited a high stereoselectivity to racemic substrate 5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1H-indene-2-carboxylic acid methyl ester (Table 13.3; Fig. 13.4b).

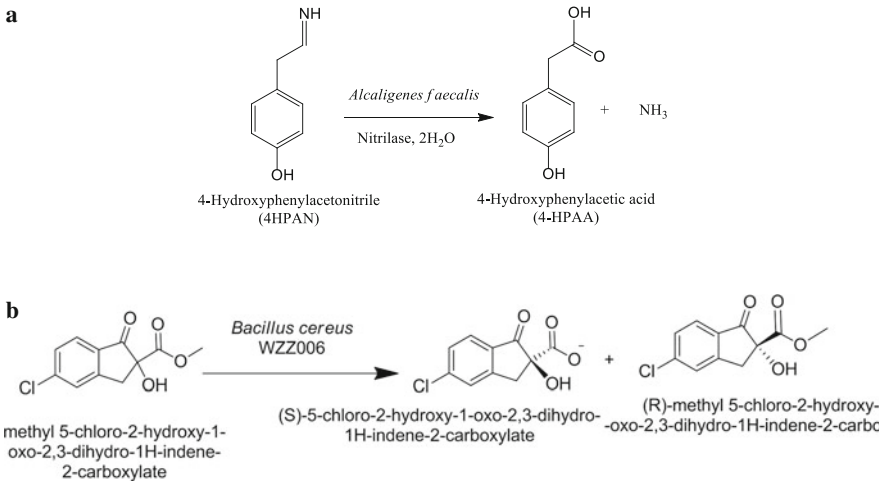

Fig. 13.4 Hydrolysis reactions catalyzed by microorganisms. (**a**): Synthesis of 4-hydroxyphenylacetic acid (4-HPAA) from 4-hydroxyphenylacetonitrile (4-HPAN) using the whole-cell arylacetonitrilase of *Alcaligenes faecalis*. (**b**): Route of resolution of (*R*,*S*)-5-chloro-1-oxo-2,3-dihydro-2-hydroxy-1*H*-indene-2-carboxylic acid methyl ester by *Bacillus cereus*

Table 13.3 Hydrolysis of some chemicals catalyzed by the biocatalyst or microbe to important products

Substrate	Biocatalyst	Target compound	Yield (%)	Reference
Racemic ester in SB-215346	*Candida antarctica* B lipase	Lotrafiban SB-214857 (orally active platelet blockers	42 99 e.e	Walsgrove et al. (2002)
Cis-3-(acetyloxy)-4-phenyl-2-azetidinone	*P. Cepacia* Lipase PS-30	Paclitaxel (anticancer compound)	45 99 e.e	Patel et al. (1994)
Racemic ethyl 2-benzyl-3-(tert-butylsulfonyl) propionate	*B. Licheniformis* Subtilisin	Renin inhibitor (angiotensin II receptors reducing blood pressure	43 >99% e.e.	Doswald et al. (1994)
2'-Deoxy-3′-thiacytidine	Cytidine deaminase from *Escherichia coli*	Lamivudine (anti-HIV agent)	76 >99.5 e.e	Mahmoudian et al. (1993)
Racemic substrate (R,S)-methyl 2-propylsuccinate 4-tert-butyl ester	Lipase M16	Intermediate (R)-2-propylsuccinic acid 4-tert-butyl Ester of brivaracetam (adjuvant in treating epilepsy)	42 97% e.e	Li et al. (2021)
Isonicotinamide	*Bacillus smithii* strain IITR6b2	Isoniazid (antibiotic to treat tuberculosis)	87.8	Agarwal et al. (2013)
Ethyl (R)-4-cyano-3-hydroxybutyate	Recombinant *E. coli* BL21 (DE3)pLysS	Rosuvastatin (cholesterol-lowering agent)	93	Yao et al. (2017)
2-Hydroxy-2-(ethoxyphenylphosphinyl)acetic acid	*Penicillium minioluteum* and *fusarium oxysporum*	2-Butyryloxy-2 (ethoxyphenylphosphinyl)ace Tic acid (bioactive compound)	52–54%, 68–93% e.e	Majewska et al. (2016)
1,3-Propanediol cyclic sulfate	*Rhodococcus* sp. CGMCC 4911	(R)-1,2-propanediol (used in the polymer, cosmetic industry)	97.2, 99% e.e.	He et al. (2015b)
1,3-Propanediol cyclic sulfate	*Bacillus* sp. CCZU11–1	1,3-Propanediol (used in the polymer, cosmetic industry)	97.4	He et al. (2015a)

13.6 Biotransformations by Whole Cells or Isolated Enzymes

The two main strategies in applying enzymes in biotransformations isolated enzymes and whole-cell systems. Cascade biotransformation could be implemented by using different forms of catalysts, including purified enzymes, immobilized enzymes, cell-free extracts, whole cells, or a mixture of them even growing cells, resting cells, immobilized cells, etc. The benefits of whole-cell biocatalysts include the following: i) the low-cost biomass cultivation is available without further downstream processes; ii) cellular structure context provides a natural environment and cofactors regeneration for many enzymes; iii) cell walls and membranes protect enzymes from reaction conditions (Wu and Li 2018).

13.6.1 Whole Cells

The whole-cell biotransformation process is divided into two types, comprising growing and resting cells. The transformations catalyzed by growing cells, the bacteria, yeasts, and fungi, are cultivated in suitable growth media to which the substrate or starting material is added to carry out the reactions. In the case of resting cell transformation, the biomass is separated by a suitable method, and cells are washed with water to remove media ingredients to which substrate is added for transformation. The inherently present enzyme either in growing cells or resting cells carries out the transformation reaction. The cell wall and the membrane acts as the barrier for the entry of the substrate or the release of enzymes in the medium. The whole-cell biotransformation is simple and cheap, and there are less chances of the enzyme getting deactivated. However, the use of whole-cell preparations of microorganisms requires expertise to handle it. The viable organisms need to be handled using the appropriate techniques to maintain the axenic cultures. The microbes before disposal need to be deactivated using chemical disinfectant or sterilized in view of biosafety (Grogan 2009). The whole cells provide a protective natural environment to enzymes, prevent loss of activity, and are significantly economical to produce than free enzymes, which require several isolations and extensive purification steps (Schmid et al. 2001; De Carvalho 2017). The advantages and disadvantages of whole cells and isolated enzymes used in the biochemical transformation reactions are stated below.

Advantages of whole cells:

1. There is no need to isolate either enzyme to its cofactor.
2. Recycling of whole cell is possible.
3. The growing cells may show higher activities to form the products.
4. The fewer by-products are generated during the transformation, and workup of products generated in the reaction is easier.
5. The most notable advantages of the use of whole cells for catalysis are that it does not require purification of enzyme, which is a tedious process.

Disadvantages of whole cells:

1. The diffusion of product or substrate takes place across the cell membrane and can be a rate-limiting step.
2. A large volume of biomass is required to catalyze the reaction.
3. In the whole-cell biocatalysis, a lower amount of product is generated due to the lower substrate concentration tolerance of the cells.
4. The cells may be susceptible to high substrate concentration and use of organic solvents.

13.6.2 Isolated Enzymes

Enzyme for biotransformation needs to be isolated in pure form from the microorganisms. The isolation and purification have been performed by an enzyme supplier companies, which were available in powdered form that could be employed as a conventional chemical catalyst. Commercial enzyme preparations are ready-to-use in nature and the possibility of reusability upon immobilization. The use of pure enzymes needs capital investment for creating facilities to purify it. The potential utility of biotransformations in the synthetic organic community has led to more companies offering a wider range of isolated enzymes than was never available previously. The isolated enzymes are usually more efficient in biotechnology than whole cells, because enzyme concentration is higher while other unwanted enzymes are absent which are generally difficult to exclude from the whole-cell biocatalysts.

Advantages of isolated enzymes:

1. The most important advantage of the isolated enzyme is better productivity.
2. The enzymes have higher activities in water, but it may exhibit tolerance to organic solvents also.
3. The recovery of product is easier compared to whole-cell biocatalyst and also the enzyme.
4. With the help of the immobilization method, the recovery of the product and enzyme is easy.

Disadvantages of isolated enzyme:

1. The cofactor recycling is necessary in case of recycling of the enzyme.
2. The enzymes are generally less stable.
3. Tedious purification techniques are required for the isolation of enzymes.
4. Although immobilization of enzyme is beneficial, its activity is reduced upon each cycle.

13.7 Immobilization of Whole Cells

The immobilization of whole cells involves the physical attachment of solid material to catalytically active cells without loss of desired biological activity (Willaert 2007). The encapsulation of immobilized cell system is termed "bioencapsulation," which is also called as "microencapsulation." Immobilization consists of microbial cells or enzymes, or other active proteins entrapped in or on the surface of a solid material enabling them to protect their catalytic activity of interest. The foremost benefit of immobilization is an easy separation of catalytic material from the reaction medium. Immobilization of whole-cell biocatalysts can improve or sustain the performance, because it can protect the enzyme from the factors that can degrade the protein or enzyme (Kisukuri and Andrade 2015). The main benefits of using this immobilization are sustained use of enzymes or microbes for selected transformation reaction, recycling of biocatalyst, and the ease in downstream processing (Liese and Hilterhaus 2013; Liese et al. 2006).

The cell immobilization confers stability to the biocatalyst preventing its degradation. The advantages and disadvantages of cell immobilization are discussed below (DiCosimo et al. 2013).

Advantages:

1. Immobilization of either whole cells or enzymes could be amenable to continuous biocatalytic and batch process.
2. Biocatalyst' reuse or recycling multiple times is possible that also reduces the cost of downstream processing.
3. It shows better stability toward various organic solvents.
4. It is possible to bring favorable alterations in pH and temperature optima.
5. Immobilization makes an easy separation of biocatalyst from the reaction mixture.
6. Co-immobilization of different biocatalysts is also possible.

Disadvantages or lacunae:

1. Immobilization reduces the catalytic activity of whole cells or even the enzymes.
2. It brings unfavorable alterations in kinetic properties.
3. Cost of operations may increase due to the additional use of carrier and fixing agent.
4. Whole cells may exhibit a lower reaction rate due to mass transfer limitations than native enzyme, which may demand more time for the biocatalytic process.

In cell immobilization, several supporting materials have been explored comprising organic, inorganic, and hybrid materials. The choice of an appropriate material depends on the type of the process. Fundamentally, the supporting material should be inert in the process and shall not interfere to reduce to the productivity generated by by-product.

Till date >100 protocols have been reported for cell immobilization, and the materials employed in these protocols could be divided into five major categories:

Table 13.4 Industrial processes employing immobilized whole cell as a biocatalyst (Polakovic et al. 2017)

Microbial culture used	Product	Immobilization method	Capacity (tons/ year)	Company
Pseudomonas chlororaphis B23	5-Cyano-valeramide	Entrapment in calcium alginate	Several	Du Pont de Nemours & co. (USA)
Alcaligenes sp., *pseudomonas* sp.	R-3-chloropropane-1,2-diol	Entrapment in calcium alginate	Unknown	Daiso co. ltd. (Japan)
Rhodococcus rhodochrous J1	Acrylamide and related products	Entrapment in polyacrylamide	>30,000	Nitto chemical Industry co., ltd. (Japan)
Brevibacterium flavum	S-maleic acid	Entrapment in j-carrageenan	468	Tanabe Seiyaku co., ltd. (Japan)
Escherichia coli B ATCC 11303	L-aspartic acid	Entrapment in polyacrylamide or j-carrageenan	700	Tanabe Seiyaku co., ltd. (Japan)
Rhodococcus rhodochrous J1	Nicotinamide (vitamin B_3)	Not specified	6000	Lonza AG (Switzerland)
Recombinant *Escherichia coli*	4-Cyanopentanonic acid	Not specified	Unknown	Du Pont de Nemours & co. (USA)
Pseudomonas dacunhae and *Escherichia coli*	L-alanine and D-aspartic acid	Not specified	61 114	Tanabe Seiyaku co., ltd. (Japan)

such as adsorption, ionic binding, covalent attachment, entrapment, and cross-linking, based on the principle employed in the immobilization (Kisukuri and Andrade 2015).

Major Classes of Cell Immobilization

1. Adsorption: The adsorption of cells on the surface of a matrix is achieved by interactions, such as electrostatic interactions, van der Waals, and hydrophobic interactions.
2. Ionic binding: This binding is based on the ionic interaction between the matrix and cells, having opposite charges on each material.
3. Covalent attachment: This comprises the presence of functional groups on the cell surfaces (such as NH_2–, –OH, –SH) that have bonding with the matrix used for immobilization.
4. Cross-linking: The cells are attached by using organic molecules, like aldehydes functioning as cross-linkers with the matrix.
5. Entrapment: The cells are captured within polymeric material or microcapsules made up of Ca-alginate, agar, κ-carrageenan, polyacrylamide, collagen, etc. (Table 13.4)

Some of the important processes catalyzed by immobilized cells as a biocatalyst used in the preparation of various important chemical products are illustrated in Table 13.5.

13.8 Recombinant Biocatalysts

The whole-cell biocatalysts have the potential to add value to the low-cost materials in environment-friendly way. The biocatalytic process may involve the native enzyme or microbes; however, for large-scale operations, the physicochemical properties need to be controlled. Industrial processes often operate under harsh conditions, like elevated pressure and temperature, extreme pH and nonaqueous solutions, and oxidative conditions that can inactivate the enzyme or microbe (Hildén et al. 2009; Luetz et al. 2008). The protein engineering strategies supporting overexpression of the protein of interest have been developed to provide tailor-made flexible and stable enzymes to adapt to specific harsh industrial conditions and reduce enzyme production costs. The optimization of parameters, like pH tolerance, solvent stability, and enantioselectivity, for better enzyme activities and specificities has been extensively improved through protein engineering techniques, leading to the rational design of catalysis and directed evolution. *Escherichia coli* is genetically well studied and the most widely used microorganism production of recombinant proteins that suits many fermentation processes. Engineered *E. coli* is a good expression system that grows fast on low-cost media and subsequently proved to be the best culture to produce recombinant biocatalyst on a large scale at a low cost (Lin and Tao 2017). Rational biocatalyst design could be achieved with the help of metagenomics, bioinformatics, protein engineering, metabolic engineering, and high-throughput screening and analysis technologies that facilitate the preparation of better biocatalyst for industrial use. These techniques have the potential to provide new and more productive biocatalyst for a wide variety of substrates and explore unknown or rare reactions in nature (Hughes 2018). Some of the important biocatalytic processes catalyzed by the various recombinant microorganisms or enzymes are illustrated in Table 13.6.

13.9 Recovery of the Product Formed upon Biotransformation

Product recovery operations from complex mixtures generated by living organisms have been developed over a period of time and are evidenced from human history. A variety of products are being produced, like alcoholic beverages, vinegar, and other food materials. Scientific developments lead to the industrial revolution that started making a variety of products, such as antibiotics, vitamins, organic acids, amino acids, proteins, fuels, other chemicals, etc. by the fermentation process. After the biocatalytic process, there are several recovery steps involved, such as centrifugation, membrane filtration, or tangential flow microfiltration to purify the product. Differential centrifugation and various filtration techniques with varied pore size

Table 13.5 Selected examples of immobilized cells applied for the synthesis of valuable compounds

Production strain	Immobilized material	Product	Yield of product	Reference
Methylocystis bryophila	Coconut coir (CC)	Methanol	48.6%	Patel et al. (2020)
Acetobacter sp. CCTCC M209061	Calcium alginate	Ethyl (R)-3-hydroxybutyrate {(R)-EHB}	> 99.0%	Wang et al. (2013)
Geotrichum candidum NCYC49	Polyacrylamide or in *Pterocladia* agar	Caprolactone	99%	Carballeira et al. (2004)
Lactobacillus animalis ATCC 35046	Calcium alginate, DEAE-sepharose	5-Fluorouracil-2′-deoxyriboside (floxuridine)	87 mg/L h 53 mg/L h	Britos et al. (2012)
Sphingomonas subarctica T7b	Polyvinyl alcohol (PVA) with sodium alginate	2-Hydroxybiphenyl (2-BHP)	267.2 mg/kg	Gunam et al. (2013)
Metschnikowia koreensis	Sodium alginate	S-alcohols	92%	Singh et al. (2012)
Penicillium citrinum CBMAI 1186	Cotton fiber Fibroin fiber Kapok fiber	Dihydrochalcones	92% 80% 93%	Ferreira et al. (2014)
Gluconobacter oxydans ZJB09113	Porous ceramics	1,3-dihydroxyacetone	177.2 g/L	Hu et al. (2017)
Cryptococcus neoformans	Amberlite XAD-2 resin	Dicarboxylic acid (DC-15)	27 mg 1^{-1} h^{-1}.	Chan and Kuo (1997)
Hansenula anomala	Acrylamide/methylenebisacrylamide (NMAAm/AAm), cross-linked polyacryloyl pyrrolidine (APr) and poly-N-methylacrylamide (NMAAm)	(+)-8S-hydroxy-4-methoxycarbonyl-9S-methyl-3oxo-octoctalin	23–52%	Saito et al. (1991)
Saccharomyces cerevisiae type II (mutant)	Sodium alginate	(−)-1-Trimethylsilyl-ethanol [(−)-1-TMSE]	>90% and > 90% e.e.	Lou et al. (2004)
Candida parapsilosis ATCC 7330	Sodium alginate	Enantiopure β-hydroxy esters	68%	Padhi and Chadha (2005)

(continued)

Table 13.5 (continued)

Production strain	Immobilized material	Product	Yield of product	Reference
Cryptococcus laurentii	Calcium alginate	(S)-1-phenylethanol	>99% e.e. and 70%	Kurbanoglu et al. (2011)
Chryseobacterium gleum	Polyethylene glycol	Protease production for keratin degradation	Complete degradation of 2% keratin	Chaudhari et al. (2013)
Recombinant *Escherichia coli*	Calcium alginate	4-Nitro-3-phenyl-butanal	97.5%, >99% e.e	Djokic et al. (2015)
Gluconobacter oxydans	Polyvinyl alcohol (PVA)	Dihydroxyacetone (DHA)	57.3 g/L	Wei et al. (2007)
Recombinant *E.-coli* BL21(DE3)/ pCDFDuet-gdh-cr	Celite-polyethyleneimine (PEI)-glutaraldehyde (GA)	(3R,5S)-tert-butyl 3,5-dihydroxy-6-isocyanohexanoate	17.5 g, diastereomeric excess >99.5%.	Wang et al. (2017)

Table 13.6 Important biocatalytic processes employing recombinant biocatalyst for the synthesis of important chemicals

Recombinant biocatalyst	Substrate	Product	Yield	References
NADH-dependent reductase (CmCR) from *Candida magnoliae* expressed in *E. coli* CCZU-K14	Ethyl 4-chloro-3-oxobutanoate (COBE)	Ethyl (S)-4-chloro-3-hydroxybutanoate [(S)-CHBE]	>99% and > 99%	He et al. (2014)
Recombinant *Escherichia coli* CCZU-T15	Ethyl 4-chloro-3-oxobutanoate (COBE)	Ethyl (S)-4-chloro-3-hydroxybutanoate [(S)-CHBE]	93–100% >99% e.e	Dai et al. (2017)
NADH-dependent carbonyl reductase (ScCR) from *Streptomyces coelicolor A3* expressed in *E-coli* BL21	Ethyl 4-chloro-3-oxobutanoate (COBE)	Ethyl (S)-4-chloro-3-hydroxybutanoate ((S)-CHBE)	93% >99% e.e	Wang et al. (2011)
Carbonyl reductase from *Rhodosporidium toruloides* expressed in *E. coli* containing R9M with GDH	Tert-butyl (S)-6- chloro-5-hydroxy-3-oxohexanoate ((S)-CHOH)	Tert-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate ((3R,5S)-CDHH)	98.9% >99% e.e	Liu et al. (2018)
Carbonyl reductase from RtSCR9from *Rhodosporidium toruloides* expressed in *E. coli* BL21 (DE3)	Tert-butyl (S)-6-chloro-5-hydroxy-3-oxohexanoate ((S)-CHOH)	Tert-butyl (3R,5S)-6-chloro-3,5-dihydroxyhexanoate ((3R,5S)-CDHH)	98–99% and > 99%	Liu et al. (2017)
Nitrilase from *Labrenzia aggregata* expressed in *Escherichia coli* BL21 (DE3)	*o*-chloromandelonitrile	(*R*)-*o*-chloromandelic acid	94.5% 96.5% e.e	Zhang et al. (2012)
NADPH-dependent carbonyl reductase gene iolS and glucose dehydrogenase gene gdh from *Bacillus subtilis* expressed in *E. coli*	Ethyl 2-oxo-4-phenylbutyrate (OPBE)	Ethyl (R)-2-hydroxy-4-phenylbutanoate [(R)-HPBE]	>98.5% e. e, 79.2%	Ni et al. (2013)
Alcohol dehydrogenase (ADH) from *lactobacillus kefir* (LK-TADH) coupled with glucose dehydrogenase (GDH) expressed in *E. coli* BL21 (DE3)	2-Chloro-1-(2, 4-dichlorophenyl) ethanone (CPE)	(S)-2-chloro-1-(2, 4-dichlorophenyl) ethanol ((S)-CPEO)	91.5%, 99.5% e.e	Chen et al. (2019)
CBR gene from *Candida parapsilosis* & GDH gene from *Exiguobacterium sibiricum* expressed in *E. coli* BL21 (DE3)	Ethyl 4-chloro-3-oxobutanoate (COBE)	(S)-4-chloro-3-hydroxybutanoate ((S)-CHBE)	97.2%, 99% e.e	Liu et al. (2015)

(continued)

Table 13.6 (continued)

Recombinant biocatalyst	Substrate	Product	Yield	References
Carbonyl reductases from *Rhodosporidium toruloides* expressed in *E. coli* RtSCR9	2-Acetylthiophene	(S)-duloxetine	92.1%, 99.9% e.e	Chen et al. (2016)
Cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 expressed in *E. coli* TOP10 [pQR239]	Bicyclo[3.2.0]hept-2-en-6-one	Lactones (−)-(1S,5R)-2-oxabicyclo [3.3.0]oct-6-en-3-one and (−)-(1R,5S)-3-oxabicyclo[3.3.0]oct-6-en-2-one	85–90%, 94–99% e.e	Doig et al. (2003)
Styrene monooxygenase from *pseudomonas* sp. strain VLB120 expressed in *E. coli* JM101	Styrene	Enantiopure (S)-styrene oxide	72.6 g L_{org}^{-1} 11 g	Kuhn et al. (2010), Panke et al. (2000)
Proline-4-hydroxylase from *Dactylosporangium* sp. RH1 expressed in *E. coli* W1485	L-proline	Trans-4-hydroxy-L-proline	41 g L^{-1}	Shibasaki et al. (2000)
Xylene monooxygenase of *pseudomonas putida* mt-2 expressed in *E. coli* JM101	Pseudocumene	Dimethylbenzaldehyde	70%	Bühler et al. (2003a, b)
Eight mammalian proteins expressed in *Saccharomyces cerevisiae*	Sterols	Hydrocortisone	0.02 g Laq^{-1}	Szczebara et al. (2003)
Engineered *Saccharomyces cerevisiae*	Amorpha-4,11-diene	Artemisinic acid	>95%	Ro et al. (2006)
BVMO from *Xanthobacter* sp. expressed in *E. coli*	Ketones	Lactones	–	Rial et al. (2008)
Nitrilase BCJ2315 from *Burkholderia cenocepacia* J2315 expressed in *E. coli* M15/BCJ2315	Mandelonitrile	(R)-(−)-mandelic acid	350 g/L), 97.4% e.e	Wang et al. (2015)

filters help efficient separation of the product, wherein no solvent is used and so are convenient in view of avoiding pollution.

The solvent extraction has been a conventional method of product extraction, where generally organic solvent is used which is immiscible in water, but the product has more solubility in it (Schügerl 1994). Owing to ease in extraction, utilization of a large quantity of solvent in industries is always deleterious to the environment, but it has been very convenient to recover proteins and enzymes without any damage (Azevedo et al. 2009).

The separation of the liquid-solid phase has been made by crystallization and precipitation processes often used in downstream processing steps (Barrett et al. 2005; Martinez et al. 2019). The processes of crystallization or precipitation can be offered by changes in pH, temperature, solvent composition, and ionic strength; even the product is soluble at high concentrations in the reaction medium. Generally, the recovery of organic acids used barium or calcium salts used in the precipitation and crystallization of products. Ammonium sulfate, having low cost and high solubility in water, is extensively used in precipitation, and further crystallization of the product, making it easy for separation, is a cheaper option of recovery (Wohlgemuth 2011). To increase the productivity of biotransformation processes, it is essential to remove the product inhibitory or degrading compounds from the vicinity of the biocatalyst or a bioreactor as soon as it is formed, which could be overcome by in situ product recovery of the product (Buque-Taboada et al. 2006). In these techniques, the extraction, adsorption, and evaporation and, in some cases, electrodialysis, precipitation, and complexation have been the most commonly employed techniques for ISPR (Freeman et al. 1993).

Polymeric adsorbers like hydrophobic polymers and functionalized polymers are good for the recovery of small molecules, where the concentration of the product is less (Garcia 1991). These polymers could be used for product separation, which is further recovered easily by bringing them into a solvent having a higher affinity for the target molecule. On the other hand, volatile products could be easily recovered simply by heating the polymer (Dafoe and Daugulis 2013). The recovery of 2,2-bis (hydroxymethyl)butyric acid (BHMB) using Ambersep®900 (OH^-) anion exchange resin resulted in ~90% of recovery from the fermentation broth. This resin strongly adsorbs the BHMB from its aqueous solution, where a high % of the product could be successfully separated (Sayed et al. 2016). The enantiomerically enriched alcohol from the prochiral ketone was recovered by the solvent extraction method. The solvent was evaporated under vacuum, and the resulting suspension was redissolved in H_2O and extracted with ethyl acetate. The organic phase was dried over anhydrous Na_2SO_4 and further purified by chromatographic technique (Barros-Filho et al. 2009; Vitale et al. 2013). Huang and Xu (2006) described the recovery process of *(S)*-mandelic acid from the racemic substrate (*RS*)-mandelic acid, where biocatalyst *Pseudomonas putida* are removed by centrifugation and the supernatant was acidified pH 5.0 and decolorized by activated carbon. The filtrate was concentrated and acidified to pH 1.0 and further extracted with ethyl acetate. The ethyl acetate layer was separated and concentrated to obtain crystals of (S)-mandelic acid.

In most biotransformation reactions, the desired end products are extracellular, where the product may be either be in a soluble or suspended state. When the whole cells are used, they have to be separated and repeatedly washed either with water or organic solvent as per needed to remove traces of nutrients. Further, the extracted product can be recovered by employing the commonly used techniques, like precipitation by salts, extraction with solvents, adsorption to ion exchangers, etc. The volatile products can be recovered by direct distillation from the medium. Biocatalysts, when used in the form of immobilized enzyme, eliminates by-product formation and avoids product degradation, However, pure enzymes are expensive and have low stability.

13.10 Conclusion

Biotransformation could be a modification in chemical compounds either by enzymes of any living entity or microorganisms. This strategy has been valuable in synthesizing molecules with better activities, which depends on the type of substrate, reaction, substrate concentration, and the ability of enzymes or microbes to transform. The amalgamation of synthetic chemical with biotransformation approaches has the potential to synthesize several compounds, which are difficult to obtain otherwise in bulk quantities in cost-effective as well as in an environment-friendly way. Owing to the abundant multienzyme systems of microbes and their exceptional abilities, transformation possesses pose several advantages over chemical synthesis processes that follow the principle of green chemistry. Mild reaction conditions required in biotransformation eventually make it environment-friendly and allow to produce *stereo*-, *regio*-, and *chemo*selective products with high conversion rates at reduced costs. Thus, the microbial transformation technique is trending in chemical process industries to produce specialty chemicals. Nowdays, there is an increasing demand for optically pure compounds in pharmaceuticals, cosmetics industries and hence, purified enzymes or microbes are being employed in several biocatalytic applications. The demand for specialty chemicals is being fulfilled by exploring newer biotransformation reactions catalyzed by the enzymes. Biotransformation has proved as an alternative to well-established chemical synthesis routes to prepare newer compounds with better activities.

13.11 Future Prospective

Making biocatalysis versatile enough to be congruent with chemical synthesis methods is a future challenge and driving force to expand through exploration of newer reactions with novel enzymes. The increasing use of sophisticated synthetic biology tools, like bioinformatics and metabolic engineering, shall be explored vigorously that can help fast screening of the compounds, strain improvement, and integrated bioprocess design, which can add value to the in an environment-friendly way.

Acknowledgments KSD and BLC are thankful to UGC-SAP and DST-FIST programs and RGSTC, Mumbai, for support at KBCNMU. GBP is thankful to the Principal, SGM College, Karad.

Declaration All the authors declare that they do not have any competing interests.

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Fermentation Strategies for Organic Acid Production

14

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Abstract

Fermentative production of organic acids by using current research can promote the economy as many countries still depend on importing these multiuse product to meet their needs. Organic acids have various applications in the food, pharmaceuticals, cosmetics, and textile industries. Besides their traditional applications, they are also used for manufacturing bioplastics. Organic acids can be produced commercially by either chemical or biological route, where the latter employs fermentation strategies. Production of organic acids by microbial processes is the preferred method due to its high productivity and environment-friendly procedure. Organic acids are low-value and high-volume products, and

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_14

therefore the use of economical substrates can boost their commercialization. The application of strategies such as recombinant DNA technology (RDT) and metabolic engineering have the capability to develop strains with high organic acid productivity. In this chapter, we will discuss industrially important organic acids (such as lactic acid, citric acid, acetic acid, kojic acid, and itaconic acid), their market potential, biosynthetic pathways, suitable substrates and microorganisms, fermentation and purification strategies, and associated challenges.

Keywords

Lactic acid (LA) · Citric acid (CA) · Acetic acid (AA) · Kojic acid (KA) · Itaconic acid (IA)

Abbreviations

AA	Acetic acid
AAB	Acetic acid bacteria
ANN	Artificial neural network
C/N ratio	Carbon to nitrogen ratio
CA	Citric acid
CAD	Cis-aconitate decarboxylase
CAGR	Compound annual growth rate
Cas9	CRISPR associated protein 9
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
CRS	Cell recycle system
DES	Diethyl sulfonates
EMP	Embden-Meyerhof-Parnas
IA	Itaconic acid
KA	Kojic acid
LA	Lactic acid
LAB	Lactic acid bacteria
MF	Microfiltration
MFS	Major facilitator superfamily
MTT	Mitochondrial tricarboxylic acid transporters
NF	Nanofiltration
PC	Pyruvate carboxylase
PQQ	Pyrroloquinoline quinone
RDT	Recombinant DNA technology
RSM	Response surface methodology
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
TCA	Tricarboxylic acid
VAM	Vinyl acetate monomer

14.1 Introduction

Fermentation technology has emerged as an environmentally friendly alternative to conventional chemical reaction-based production. Using microbial biosynthetic pathways, chiral selection of the end products such as lactic acid becomes more feasible as compared to the chemical route of synthesis. Continuous efforts have been made to improvise the production process of different metabolites of microbial origin, which have large industrial applications. Organic acids are one such building block having a variety of commercial uses. Citric acid produced from *Aspergillus niger* is produced as microbial acid, having a market of 2.39 million tons in 2020, which is expected to reach 2.91 million tons by 2026 with a CAGR of 4.0%, according to a global citric acid market outlook. The cost of production can be reduced by bioprocess advances in production strategies. These include the development of high-yielding strains, deployment of metabolic engineering, optimization of production and purification methods, proper selection of modes of fermentation, and integration of production and downstream processing. This chapter summarizes the recent developments in the bioprocessing strategies of some organic acids with huge commercial applications, such as lactic acid, citric acid, acetic acid, kojic acid, and itaconic acid. Their biosynthetic routes in different microorganisms have been investigated to understand the scope of possible interventions for enhanced production. The production process limitations have been discussed in light of medium, pH, temperature, aeration, and agitation requirements. The possibility of agro-waste material utilization as a substrate has been analyzed to integrate the production process in a circular economy. The purification process involves a significant component of the total capital requirement. Therefore, the same has been reviewed for deciphering the critical issues related to process efficiency. A comprehensive analysis of the current trends in organic acid fermentation strategies has been presented in the following sections, and the future scope of development has been proposed.

14.2 Lactic Acid (LA)

Lactic acid (LA) is a low-molecular-weight organic acid produced on a large scale either through chemical or biological methods (fermentation). It has several applications in the food industry, as a flavor enhancer and pH regulator and used for fortification; in the chemical industry, as a green solvent, neutralizer, and slow acid-releasing agent; and in the pharmaceutical industry, used for mineral preparation, intravenous solution, and dialysis solution and also used in the manufacture of biodegradable plastic (Polylactic acid) (Abedi and Hashemi 2020; Komesu et al. 2017; Wee et al. 2006). A racemic mixture of DL-LA is synthesized by a chemical route, which can increase the purification cost. Therefore, microbial fermentation is the preferred method with pure D or L LA, depending on the variety of microorganisms. Microbial fermentation provides several benefits over chemical processes, such as posing fewer environmental problems and inexpensive substrates

with low energy requirements (Komesu et al. 2017). The most commonly used substrates are carbohydrates obtained from corn starch, sugarcane molasses and tapioca starch, and other locally available substrates. The predominant problem associated with biomolecules like LA production is suitable substrates with low price and not competing with food-grade material. Lignocellulosic biomass has the great potential to provide a sufficient amount of substrate for the production of biomolecules. However, it must be pretreated and hydrolyzed into fermentable sugar so that LA-producing microorganisms can utilize it efficiently and produce LA (Kim et al. 2020).

14.2.1 Biosynthetic Pathways for LA Production

LA can be produced from glucose via glycolytic pathway/pentose phosphate pathway, where LA is obtained as the primary end product (homolactic acid metabolism) or via phosphoketolase pathway (heterolactic acid metabolism), where other by-products such as AA and ethanol are formed in addition to LA as shown in Fig. 14.1. (Yang et al. 2020). Homolactic acid metabolism occurs in substrates, which are rapidly utilized by microorganisms, and more than 90% of sugars being metabolized into LA. However, heterolactic acid metabolism is observed under aerobic conditions or during the metabolism of less rapidly utilized sugars, such as galactose or maltose (Papagianni 2012). In homolactic acid metabolisms, microorganisms possess the aldolase enzyme and convert hexoses and pentoses via the glycolysis pathway (EMP pathway) or pentose phosphate pathway into lactic acid. Homofermentative microorganisms produce two moles of LA from one glucose mole with a theoretical yield of 1.0 g/g.

14.2.2 LA Producing Microorganisms

LA is produced through bioprocess by different microorganisms such as bacteria and fungi, which can utilize various carbohydrates. Microalgae and cyanobacteria are photosynthetic microorganisms that can also produce LA without the supply of carbohydrates. The selection of suitable strain is very crucial for producing optically pure LA with high productivity. The large-scale LA production is mostly performed by employing the LA-producing bacteria, classified into four major groups: Lactic Acid Bacteria (LAB), *Corynebacterium glutamicum*, *Escherichia coli*, and *Bacillus* sp. Among these, LAB is mostly used bacteria for LA production due to their tolerance to low pH, high yield, and high LA productivity. The LAB mainly consists of four genera, *Streptococcus*, *Lactobacillus, Leuconostoc*, and *Pediococcus*. These LAB are used directly as wild-type genetically stable strains screened from various natural sources or engineered strains obtained through mutagenesis and metabolic engineering. The most commonly used mutagens are nitrosoguanidine, ethyl methyl sulfonate, or UV radiation exposed to microbial populations for different periods to get the desired level of mutagenicity. The use of these mutagens creates random

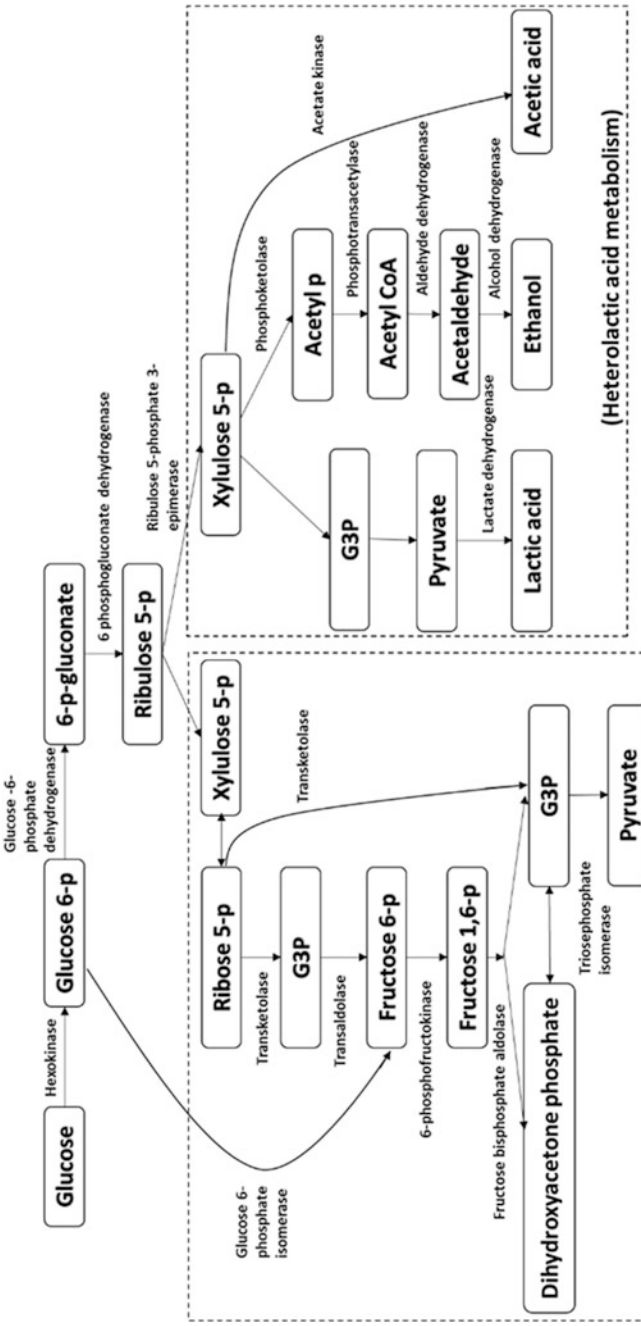

Fig. 14.1 Biosynthetic pathway of LA

mutations in the DNA of the selected cells. However, metabolic engineering is the targeted genetic modification of the microbial cells to regulate the gene expression, alter the cellular proteins level, and redirect the metabolic flux. LA can be produced from various substrates using different modes of fermentation such as batch, fed-batch, and continuous. Some of the important microorganisms with high yield and productivity are mentioned in Table 14.1.

14.2.3 Substrates for LA Production

Various feedstocks can be employed for LA production. The main factors associated with low-cost LA production are the availability of cheap substrate and fermentation process, which accounts for approximately 40–70% of total production cost. Its production mainly depends on the sugars and starch derived from food materials, such as corn, beets, and sugarcane. Therefore, cheap substrates, such as food waste, lignocellulosic biomass, or microalgae, have been strongly proposed as substrates for LA production. Many scientists worldwide are working on LA production from lignocellulosic biomass, and many studies have been reported where these substrates have been used for LA production. However, they are still unable to produce LA economically from lignocellulosic biomass. The main challenges associated with lignocellulosic biomass are its pretreatment and the cost of enzymes involved in hydrolysis. The pretreatment of lignocellulosic biomass is necessary because it separates all the constituents (lignin, cellulose, and hemicellulose) and allows the enzymes to efficiently catalyze the conversion of cellulose and hemicellulose into fermentable sugars.

The most commonly used pretreatments are mechanical (milling and grinding), chemical (acid, alkali, and some organic solvents), physicochemical (hydrothermal, steam explosion, ammonia fiber explosion, wet oxidation, etc.), and biological techniques (bacterial and fungal pretreatment). The acidic pretreatment helps to remove the hemicellulose from the lignocellulosic biomass, whereas the alkaline pretreatment enhances the delignification process. In the acidic pretreatment, a low acid concentration at high temperature or strong acid at low temperature is commonly used for lignocellulosic biomass pretreatment. Although immensely useful in hydrolyzing hemicellulose fraction, chemical pretreatments increase the process cost and generate toxic by-products, such as furfural, 5-hydroxymethyl furfural, and weak acids, which inhibit the microbial conversion processes. Moreover, these chemical pretreatment residues also cause pollution and damage the ecosystem when released into the environment.

The biological pretreatment methods are more suitable than physical and chemical techniques because of their cost-effectiveness and environment-friendly nature. However, its application is restricted due to the long pretreatment time. Novel biomass pretreatment methods have also been developed using green solvents, such as ionic liquids and deep eutectic solvents under mid conditions.

Table 14.1 List of some important microorganisms reported for high yield and productivity of LA

Microorganisms used	Substrate used	Pretreatment	Mode of fermentation	Production titer	Yield	Productivity	Reference
Lactobacillus pentosus FL0421	Corn stover	14% (w/w) NaOH	Fed-batch SSF	92.30 g L^{-1}	0.66 g g^{-1}stover	1.92 g L^{-1} h^{-1}	Hu et al. (2016)
Lactobacillus delbrueckii ssp. *delbrueckii* CECT286	Orange peel wastes hydrolysate	–	Batch SHF	–	0.88 g g^{-1} of substrate	6.72 g L^{-1} h^{-1}	De la Torre et al. (2019)
Pediococcus acidilactici PA204	Corn steep powder	12% (w/w) NaOH	Batch SSF	92.01 g L^{-1}	0.77 g g^{-1}stover	1.28 g L^{-1} h^{-1}	Bastard et al. (2020)
Pediococcus acidilactici PA204	Corn steep powder	15% NaOH	Batch SSF	104.11 g L^{-1}	0.69 g g^{-1}stover	1.24 g L^{-1} h^{-1}	Bastard et al. (2020)
Bacillus coagulans	Bakery waste hydrolysates and lucerne green juice	–	Batch fermentation mode	62.2 g L^{-1}	0.57 g g^{-1}bakery waste	2.59 g L^{-1} h^{-1}	Alexandri et al. (2020)
Bacillus coagulans	Bakery waste hydrolysates and lucerne green juice	–	Continuous fermentation	–	–	11.28 g L^{-1} h^{-1}	Alexandri et al. (2020)
Lactobacillus delbrueckii ATCC 11842	Beechwood	Organosolv pretreatment	Bach SSF	62 g L^{-1}	0.69 g g^{-1} of biomass	0.86 g L^{-1} h^{-1}	Karnaouri et al. (2020)
Lactobacillus delbrueckii ATCC 11842	Pine	Organosolv pretreatment	Bach SSF	36.4 g L^{-1}	0.40 g g^{-1} of biomass	0.51 g L^{-1} h^{-1}	Karnaouri et al. (2020)

14.2.4 LA Production Strategies

Several studies have been performed to increase LA productivity and bioprocess viability on a large scale. There are mainly four challenges (purity of LA, low tolerance of microorganism, utilization of carbon source, and optimization of fermentation parameters) during LA production. Some of the recent techniques and advances for increased production of LA are discussed here.

14.2.4.1 LA Production in Co-culture

Co-culture is a cultivation technique in which two or more microorganisms are grown to produce the desired product with high yield or productivity. Co-culture is mainly essential when one cell culture cannot grow alone, and one cell population promotes the growth of other cell population. Some LA-producing bacteria, due to their heterofermentative nature, produce other by-products including AA and ethanol, which decreases productivity and increases the cost of production. In some cases, the co-culturing of a homofermentative bacteria (which has a significantly higher growth rate), with heterofermentative bacteria, induces heterofermentative bacteria to follow a different metabolic pathway to convert substrate into more LA and consequently reduces the by-products accumulation. Zhang and Vadlani (2015) performed the co-culture study of *Lactobacillus brevis* ATCC 367 with *L. plantarum* ATCC 21028 and reported the increase in LA yield from 0.52 g g^{-1} from a propeller hydrolysate to 0.88 g g^{-1} and also observed a decrease in by-product concentrations (Zhang and Vadlani 2015). In a similar study, Cui et al. (2011) studied the LA production from corn stover hydrolysate using a mixed culture of *L. rhamnosus* and *L. brevis* (Cui et al. 2011). They observed that the LA yield (0.70 g g^{-1}) was increased by co-culture fermentation, which was 29.6% and 18.6% higher than the yields obtained by single cultures of *L. brevis* and *L. rhamnosus,* respectively. Therefore, co-culture fermentation is helpful in enhanced LA production and reduces the by-product formation and, consequently, improves the economics of the process by reducing the separation and purification cost.

14.2.4.2 LA Production by Strain Improvement Using Genetic Modification and Metabolic Engineering

Metabolic engineering is a powerful technique in biotechnology, which allows developing compelling strains that can tolerate low acidic environments and grow in a wide variety of substrates with enhanced LA production and reduced by-product formation. The most common problems observed in LA production are the reduced acid tolerance of producing strains and utilization of the limited amount of substrate. Lactic acid bacteria (LAB) are a collection of bacteria that can naturally produce LA as a primary product. However, LA's optical and chemical purity may vary because of the inefficient utilization of substrate through heterolactic acid metabolisms (Eş et al. 2018). Therefore, this emerging technology's role becomes more critical to redirect the metabolic pathway for efficient utilization of substrate to LA production with enhanced chemical and optical purity.

Metabolic engineering involves two approaches, i.e., rational engineering and evolutionary engineering. Rational metabolic engineering includes pathway redirection, increased gene expression, and heterologous gene expression. The evolutionary metabolic engineering approach involves classical strain improvement, shotgun-mutagenic approach, genome shuffling, whole genome amplification, and multiplex automated genome engineering (Upadhyaya et al. 2014). Each gene has a specific metabolic function in the host microorganisms. Therefore, a complete analysis of transferred genes and their effect on host microorganisms is essential for genetic improvement to become successful. Kadam et al. (2006) developed mutant strains of *L. delbrueckii* (NCIM 2365) by acclimatization and ultraviolet mutagenesis, which increased the LA production by 10% than the LA production by wild-type strain (Kadam et al. 2006). In another study, Ozaki et al. (2017) developed a metabolically engineered strain *Schizosaccharomyces pombe* for enhanced LA production using a CRISPR-Cas9 system by disrupting the genes encoding pyruvate decarboxylases, L-LA dehydrogenase, and alcohol dehydrogenase enzymes, thereby reducing the ethanol production (Ozaki et al. 2017). The engineered strain efficiently consumed glucose and cellobiose and produced D-LA at 25.2 g/L and 24.4 g/L with a yield of 0.71 g D-LA/g glucose and 0.68 g D-LA/g glucose, respectively. Tian et al. (2021) also used metabolic engineering along with adaptive evolution strategies. They developed a high optical purity L-LA-producing strain of *L. paracasei* through CRISPR-Cas9 gene-editing system, which could produce a significant amount of LA (221 g/L) even at high temperatures (45 $^{\circ}$ C) with a productivity of 7.5 g/L/h and yield of 0.96 g/g (Tian et al. 2021). Therefore, metabolic engineering helps improve the LA productivity and yield and enhances its quality in the fermentation broth.

14.2.4.3 LA Production in Immobilized Bioreactor

In bioprocessing, immobilization is the technique of fixing biocatalysts such as enzymes or cells, which catalyzes the particular biochemical reactions to produce a specific product. The primary purpose of immobilization is to allow the reusability of the valuable biocatalyst and reducing its activity loss. The immobilization is mainly performed to obtain more viable bioprocesses with higher productivity. In some cases, cells are genetically modified or metabolically engineered for efficient production of LA. Thus, these cells must be reused repeatedly to reduce the production's overall cost, since these modifications contribute to a significant amount in the total cost of production. The LA productivity depends on the type of immobilization technique (adsorption, absorption, entrapment, gel encapsulation, cross-linking, and covalent bonding) and support material used for immobilization. These immobilization techniques and support materials are used in a particular type of bioreactors to achieve a high yield of LA. Sirisansaneeyakul et al. (2007) immobilized the *Lactococcus lactis* cells by encapsulating the alginate membrane's microcapsules and studied the LA in a packed bed reactor (Sirisansaneeyakul et al. 2007). They reported that LA's volumetric productivity increased from 1.8 g/L/h to 4.5 g/L/h when the broth was recycled and passed through the packed bed of encapsulated cells. In another study, Wang et al. (2020) optimized the fermentation parameters for LA production by immobilized *L. pentosus* ATCC 8041 (Wang et al.

2020). They obtained maximum LA yield (0.938 g/g) and productivity (2.213 g/L/h) by the optimization study. The optimized conditions include pH 5.60, 2.0 mm bead diameter, initial glucose concentration as 115.3 g/L, and biomass concentration as 3.982 g/L (biomass per L hydrogel).

14.2.4.4 LA Production by Cell Recycle System

Cell recycle system (CRS) allows the fermentation to occur at a very high cell density and thus improves the process productivity. These CRSs are generally used with a stirred tank reactor. After the fermentation, the production broth containing the desired product is passed through the cross-flow filtration, where cells are retained and recirculated back into the fermenter to continue the production process, while the permeate contains the LA which is collected in a separate vessel. Most of the time, the LA is produced by genetically modified strains. Therefore, the recovery of these cells using CRS enhances economics by reducing the production's overall cost. Other limitations, such as pH drop due to end-product inhibition, are also reduced by using CRS. However, some of the disadvantages are also associated with CRS, such as frequent sterilization of the continuous system and loss of biocatalytic activity due to shear stress. Thus, it becomes essential to optimize the CRS, especially in the case of large-scale production. González-Vara et al. studied LA production in chemostat cultivation by recycling culture media and cells (González-Vara y R et al. 2000). They reported that the highest LA productivity (138 g/L/h) could be achieved in a continuous fermentation with a cell recycling system. The cell recycling system is also helpful in reducing the by-products (AA and ethanol) formation during LA fermentation and thus further improving LA's productivity. Abdel-Rahman et al. (2016) studied the continuous fermentation of xylose with cell recycling for the production LA using *Enterococcus mundtii* QU 25. They reported that L-LA productivity could be increased to threefold under optimized pH conditions from 1.94 to 5.33 g L^{-1} h^{-1}, and it was further increased to 6.15 g L^{-1} h^{-1} when the feed was optimized (Abdel-Rahman et al. 2016).

14.2.4.5 LA Production Using Simultaneous Saccharification and Fermentation (SSF)

Saccharification is the process in which polysaccharides are hydrolyzed into simple sugars so that LA-producing microorganisms can consume sugars very efficiently to produce more LA. In conventional LA fermentation from polysaccharides, both enzymatic hydrolysis and fermentation take place in separate reactors. However, SSF is a process where saccharification of carbohydrates and microbial fermentation occurs in a single step. The SSF is an effective strategy for improving LA production because it avoids substrate inhibition and reduces the processing time. Wang et al. (2015a, b) studied an open SSF of white rice bran for LA production by a thermotolerant strain of *Bacillus coagulans* LA-15-2 (Wang et al. 2015b). They reported that in batch fermentation under SSF conditions, LA was obtained with a maximum titer of 17 g L^{-1}, the productivity of 2.79 g L^{-1} h^{-1}, and yield reached 98.75%. Van der Pol et al. (2016) also investigated the SSF process of

lignocellulose-rich sugarcane bagasse for LA production by *Bacillus coagulans* DSM2314 with the productivity of 2.54 g/L/h (van der Pol et al. 2016).

Moreover, the SSF allows LA production with a higher titer. Chen et al. (2019) studied batch and fed-batch SSF of dilute ethylenediamine (thermochemical) pretreated rice straw for LA production (Chen et al. 2019). They reported that under fed-batch fermentation, the LA titer could be increased from $63.5 \pm 3.0\,g\,L^{-1}$ to $92.5\,g\,L^{-1}$ and yield from 296.8 g Kg^{-1} to 578.1 g Kg^{-1}. In the SSF process, the glucose consumption rate is the crucial factor that decides the LA productivity in the fermentation broth. If the glucose consumption rate is less than its production rate, it leads to glucose accumulation in the fermentation broth, which is not desirable and leads to substrate inhibition. Also, glucose accumulation reduces the consumption of other sugars, such as xylose by microorganisms. Thus, the glucose consumption rate should be more than its consumption rate for higher LA productivity. Therefore, a kinetic study of microorganisms must be performed to determine the glucose consumption rate before LA's production.

14.3 Citric Acid (CA)

Citric acid is a distinct major highly vital innate organic acids and is harmless and benign, palatable having a sour taste. From the ancient word citrus, the name of the natural organic acid CA is derived and procured naturally from the living cells as it is found as an intermediary in the tricarboxylic acid cycle, which happens to be found in the metabolism of all the aerobic organisms (Swain et al. 2011). This naturally found organic acid is colorless and odorless and exists as white powder-crystalline in nature. CA is an emulsifier, preservative, sequestrant, acidulant, buffering agent, and flavoring extensively used in many industries, such as pharmaceutical, cosmetics, nutraceuticals, beverages, and food industries (Verhoff and Bauweleers 2000). Therefore, such a promising compound's daily consumption is in very high demand (Radwan et al. 2010; Soccol et al. 2006; Vasanthabharathi et al. 2013). It is surveyed that approximately 70% of the entire CA produced is employed by the food and beverage industries, and remnant 30% is utilized by the medical, pharmaceutical, chemical, and several variant commercial enterprises (Soccol et al. 2003). The most wide-reaching vendee of CA is the food vocations owing to its many excellent characteristics (El-Hussein et al. 2009; Yalcin et al. 2010). Inside the food exercising vocations, CA utilization is done because of its antioxidant competency to conserve eatables or used as an acidifier to increase the aroma and flavors of food, ice cream, and fruit juices. CA is utilized in the role of an antioxidant in the pharmaceutical industries to conserve vitamins, blood preservatives, pH corrector, iron tablets for the source of iron, cosmetics, and ointments preparations (Max et al. 2010).

Furthermore, this organic acid is utilized as a foaming agent for treating and softening textiles in the chemical industries. As a phosphate substitute, citric is also utilized in the detergent industry due to its less eutrophic effect (Max et al. 2010). The evolution of CA production climbed to a great extent in the last decade because

of its demand and proper knowledge of biotechnology, which uses the fermentation technique and applies the best product purification and product recoveries.

14.3.1 Biosynthetic Pathways for CA Production

In the last few years, CA accumulation and production knowledge based on its biochemistry have been reported by many research groups (Max et al. 2010; Papagianni 2007; Show et al. 2015). Several studies demonstrated that various biological factors like dissolved oxygen; the concentration of carbon source, hydrogen ions, and subprime concentrations of trace metals and phosphate were responsible for CA overflow but diversified between individuals' strains (Kristiansen and Sinclair 1979). The bio-fabrication of the CA initiates with the absorption of sugar, which goes through glycolysis for the production of two molecules of pyruvate (Fig. 14.2). Molecules of pyruvate generated through glycolysis are then readily entrusted to the inner membrane of mitochondria thenceforth and in the matrix. Their oxidization is then done and then associated with the coenzyme A (CoA) to produce CO_2, NADH, and acetyl-CoA. Newly formed acetyl-CoA and oxaloacetate combine to form citrate. On the contrary, the next possible thing is that pyruvate generated via glycolysis is carboxylated with pyruvate carboxylase to produce oxaloacetate (an intermediate of Krebs cycle) to drench up the quota of oxaloacetate in the TCA cycle for the metabolism of the acetyl-CoA. CA hereby synthesized because the oxaloacetate and acetyl-CoA are now transformed through a series of reactions into carbon dioxide (two molecules) and regenerates oxaloacetate (four molecules) again. Hence, in every round of this cycle, an acetyl-CoA molecule enters, and the formation of carbon dioxide (two molecules) and ATP take place, and also, utilization of an oxaloacetate molecule is done to produce citrate (Behera 2020).

14.3.2 CA-Producing Microorganisms

The synthesis of CA happens to be earlier accomplished by various physical and chemical modes, but such modes were complicated, and neither economically favored nor environmentally friendly (Kumar Gupta et al. 2016; Yin et al. 2017; Yu et al. 2018). Fabrication of CA with the help of microbial fermentation is the most feasible way compared to plants and animals. Many microbes produce CA like *Penicillium* (Wehmer 1893), *Candida*, *Yarrowia*, *Hansenula*, *Torula*, *Kloeckera*, *Saccharomyces*, *Pichia*, *Zygosaccharomyces*, *Torulopsis*, *Bacillus licheniformis*, *Corynebacterium* spp., and *Arthrobacter paraffinens* (Vandenberghe et al. 1999), but the organism of fondness for the production of CA at the commercial level is *Aspergillus niger* (Alnassar et al. 2016; Selvankumar et al. 2014; Singh et al. 2016). *A. niger* grows profusely in a nutritional medium of pH range between 2.5 and 3.5 and produces a huge amount of CA (Currie 1917). It also has the capability to use a large variety of inexpensive agro-waste substrates as a raw material because of its high polymer enzyme degrading system (Papagianni 2007). In contemplating getting

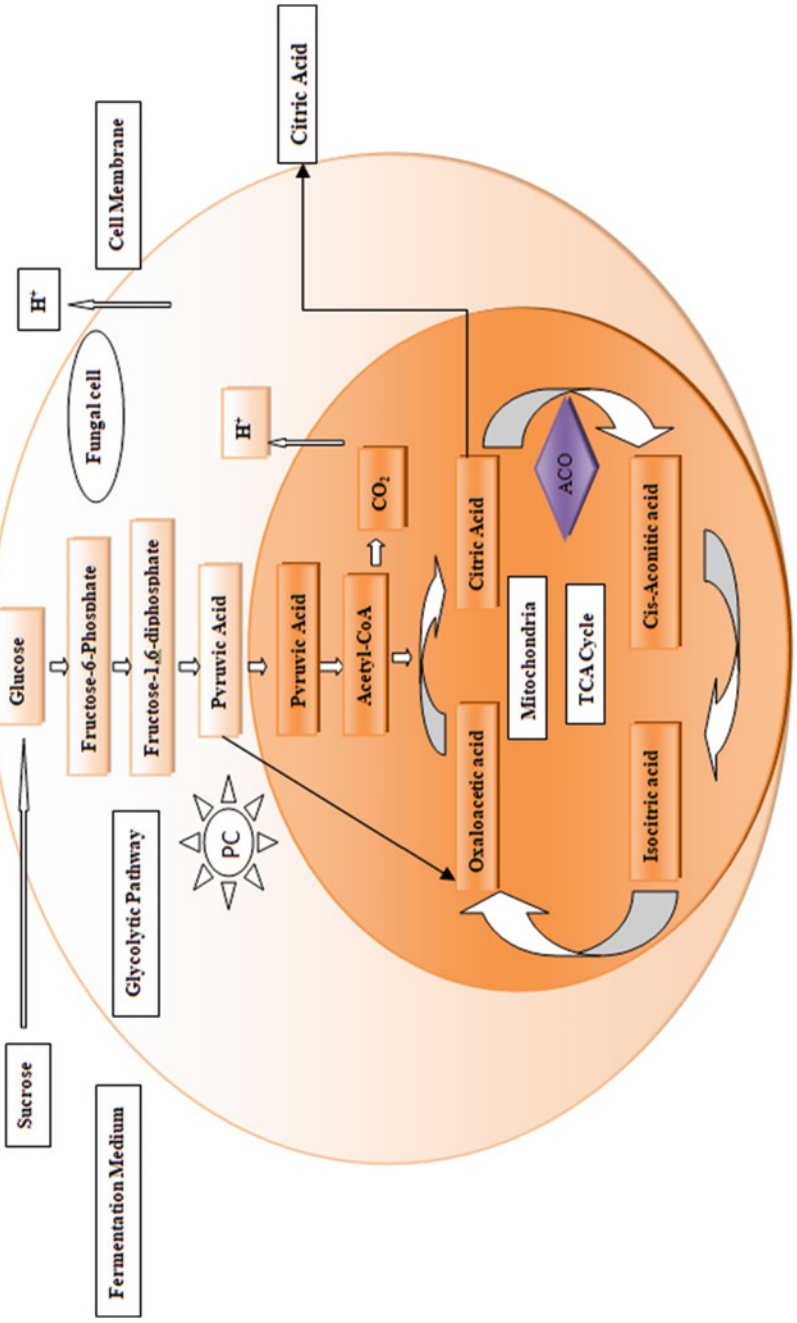

Fig. 14.2 Citric acid production by *Aspergillus niger* (*ACO* aconitase, *PC* pyruvate carboxylase), with little amendment (Soccol et al. 2006)

Table 14.2 Comparative study of citric acid produced by utilizing a variety of raw materials

Microorganisms	Media/substrate	Citric acid produced (g/L)	Reference
A. niger (KA88)	Corn cobs	138.24	Addo et al. (2016)
A. niger (GMCC 5751)	Corn fluidified medium	151.67	Wang et al. (2015a)
Y. lipolytica CBS 2073)	Glycerol (crude)	10.00	Ferreira et al. (2016)
Penicillium oxalicum	Potato dextrose media	530.00	Li et al. (2016)
A. niger (GCB 117)	Molasses (cane)	14.17	Iqbal and Utara (2015)
A. niger (FUO2)	Malted sorghum of peel of Casanova	1.93	Adeoye et al. (2015)
Y. lipolytica (K-168)	Medium based on juice of carrot	80.53	Urak et al. (2015)
Saccharomycopsis lipolytica	Oil of canola	152.30	Good et al. (1985)
Y. lipolytica (NRRL Y-1095)	Glycerol	59.00	Avila Neto et al. (2014)
A. niger (MTCC 281)	The dry and pulpy residue of apple	4.6 g/100 g of pomace	Kumar et al. (2010)

optimum yield through fermentation with decreased cost, a substrate is the most crucial factor for the productivity of CA (Podgórski et al. 2004). Table 14.2 shows the use of various kinds of raw materials for CA production via different microorganisms.

14.3.3 CA Production by Strain Improvement

Improvement of the producing strain is an important means to heighten up CA production. Strain betterment by way of the parasexual cycle was fundamentally reported in 1953 (Pontecorvo et al. 1953). CA produced by haploid parents was much less than the diploids one (Das and Roy 1978). Genetic manipulation through protoplast fusion in *A. niger* for increased CA production is also a beneficial technique (Kirimura et al. 1988). Passage and single spore techniques are also conventional methods for choosing improved strains (Soccol et al. 2006). With the help of some physical and chemical mutagens, induction of mutation to achieve modified metabolism can also be practiced to improve strain (Swain et al. 2011). Gamma radiation and UV radiation are the most common physical mutagens used for mutation procedures for enhanced CA production (Pelechova et al. 1990). MNNG, NMU, diethyl sulfonates (DES), and ethidium bromide aziridine are some very popular chemical mutagens used for strain improvement (Musilkova et al. 1982). For the distinguishing of the mutated strain, enzymatic diffusion zone

analysis is carried out, followed by screening for improved production capability (Prasad et al. 2014).

14.3.4 CA Production Strategies

Toward industrial-scale CA production, fermentation is the most compatible mode. Generally, three types of fermentation are practiced for the synthesis of CA.

14.3.4.1 CA Production by Submerged Fermentation

Presently submerged fermentation has been adapted as the most extensively used fermentation technique (Show et al. 2015). In this technique, microorganism nurtures all over the nutrient broth medium on the inmost the nutrient bedrock and dissolved carbon sources (Lingappa et al. 2002; Swain et al. 2011). The whole fermentation process is executed inside a bioreactor (aseptic) made up of a blended flask and a system for air supply, consolidated with a facility for heat exchange, like water jackets and snake tubes. The use of impeller blades and sparger is done for the maintenance of the culture conditions. Usually, fermentation for CA is wind up between 5 and 12 days, based on the procedure circumstances (Soccol et al. 2006). Some benefits of using submerged fermentation techniques are the reasonable command of the procedure and utilization of a broad dimension of substrates (Max et al. 2010). This technique delivers good maintenance, lower labor capital, costs and risk of contamination, and higher yields (Sawant et al. 2020). The disadvantage associated with the submerged fermentation is the building of froth during the process, which the use of antifoaming agents can avert.

14.3.4.2 CA Production by Surface Fermentation

Surface fermentation is the inception of CA's fermentation procedure designated by less produce, foam-free, and diminished energy consummation (Kiel et al. 1981). However, its maintenance cost is much higher and labor-intensive compared to submerged fermentation, because of the cleaning procedure of the trays, pipes, and walls of the reactor (Drysdale and McKay 1995). The whole process is also very susceptive to fluctuations in the nutrient media composition (Benghazi et al. 2014). Fermentation is executed in the cubicle, utilizing various trays (mostly made up of stainless steel) and systemized in shelves (Bauweleers et al. 2014). This process is an immobilized batch fermentation process, and it requires 8–12 days to be completed. Firstly, in the sterilization of the nutrient media present in the trays, inoculation is done with spore, and then incubation is done at 28–30 °C (Marzona 1996) for 24 h. An immense quantity of heat is produced at the time of fermentation, so tremendous aeration is required for maintenance of the temperature. The fermentation chambers ought to be maintained in aseptic conditions; as of yeasts, *Aspergillus* and *Penicillia* are some common contaminants that can easily contaminate the whole process. When the process is completed, recovery of CA is done through separation and washing of the mycelia mats with the help of boiled water (Max et al. 2010).

14.3.4.3 CA Production by Solid-State Fermentation

In this process, microorganisms are grown in less water accommodating intricate stuff that functions both as sources of nutrients and as physical support (Pandey 1992). The fermentation process takes 4–5 days for completion (Drysdale and McKay 1995). The solid substrate utilized is dampened nearly 70% prior to fermentation. The pH requirement is around 4.5–6.0, and the temperature is maintained at 28–30 °C. The energy requirement and risk of contamination are very low in this process, and also downstream processing of CA can be done quickly. The main advantage is that this process provides a higher yield than submerged fermentation, and also substrates do not need some sort of preoccupational therapy because the mode is not very susceptive to the trace elements present (Berovic and Legisa 2007). The deficit of this process is that it is difficult to control process parameters, and also, there is a problem with the heat generation. The available nutrients are not completely utilized because of insufficient oxygen supply and limited heat displacement in the substrate (Kapilan 2015).

14.3.4.4 CA Production by Metabolic Engineering

The increase dexigency for CA has fascinated many research scholars in developing the believable strain that can excess produce CA by manipulating the genome. Many hit and trial methods are run to increase the production by renovating the genes and metabolic pathways that control CA production (Ruijter et al. 1997, 1999; Yin et al. 2015). CA production by *A. niger* utilizing corn starch as a primal material noticed that at the end of the fermentation isomaltose stay behind is unutilized (Wang et al. 2016), and it affects the large-scale production and also the profits. The isomaltose (residual sugar) is made by α-glucosidase, and the deletion of agdA gene, which encodes α-glucosidase, decreases isomaltose concentration up to 88.2% and increases the CA production by 16.9% (Wang et al. 2016).

14.3.4.5 CA Production by Genetic Engineering

Genome editing of the metabolic pathway of *A. niger*, appropriately amends the connatural genome to reconstruct the anatomical conduct and provoke the biogenesis of secondary metabolites and proteins, which can allow newly formed strains of fungi with uplifted production of a specific compound (Ulaganathan et al. 2017). A gene can be assembled or deleted by applying this technique and upregulated or downregulated within the organism at a specific site. A very dominant technique for genome editing is CRISPR-associated protein system (Hsu et al. 2014; Jinek et al. 2014). Apart from helping the chromosome designing of *A. niger*, this system also eases the genome's manipulation by dissecting the target site. Using this technique, disruptions in genes have been developed in the *A. niger* in an advanced-performance modus, thereby enhancing the system's metabolic engineering (Tong et al. 2019). An essential enzyme orotidine-50-decarboxylase gene pyrG is tangled in uridine's biosynthesis (Van Hartingsveldt et al. 1987). It has been discovered that downregulation and gene disruption of pyrG notably enhances CA production via submerged fermentation (Zhang et al. 2020).

14.3.5 CA Purification Strategies

At the closing of the fermentation, the recovered product holds numerous unwanted by-products like mineral salts, mycelium, proteins, other organic acids, and several other impurities along with CA. So, it becomes mandatory to purify CA from the product obtained from a fermentation medium, which depends upon the techniques, and the substrates used (Grewal and Kalra 1995). Firstly, mycelia are set apart from the fermentation broth by either centrifugation or filtration as it holds up to 15% of the formed CA. In the surface fermentation method, the product acquired is drawn apart from the trays, and then boiled water is utilized to obtain the excess CA to mycelia. Although at the end of submerged fermentation, CA separation from the fermented broth is more laborious. As a first step, the fermented broth containing mycelia is warmed up to 70 °C for approximately 15 min, and after that, filtration is done by using belt discharge filters or centrifugation or a rotating vacuum drum. Washing and pressing of mycelia can also be done to get the extra CA present in the media (Max et al. 2010). The oxalic acid formation has also been observed during CA production, and it can be pulled out by shooting up the pH up to 3.0 with the help of calcium hydroxide at temperatures 72–75 °C (Sawant et al. 2020). As the result of which calcium oxalate is made up, this is antecedently precipitated and removed through filtration or centrifugation. In the original solution, CA is present in calcium salt, which can be purified by several techniques like solvent extraction, precipitation, electro-dialysis, liquid membranes, ultrafiltration, and adsorption.

14.3.6 Challenges and Future Scope for CA Bioprocessing

CA is one of the enormously utilized organic acids. Economically favorable CA production is dependent on several factors, like substrate used, microorganism used, control over bioreactor used, employed fermentation technique, the recovery process, and quantification techniques. Delineating the suitable bioreactors with suitable process control can be achieved to improve the process of CA production. Noteworthy efforts are still to be made in strain improvement techniques. Elimination of such obstacles will lead to enhanced CA production and will be economically favorable for the industries.

14.4 Acetic Acid (AA)

Acetic acid (CH_3COOH), also known as methanoic acid, is an important colorless organic acid, having more comprehensive industrial applications. It has a global demand of approximately 15 million tons per year, and an average growth rate of 5% per year is estimated, especially in Asia (Deshmukh and Manyar 2020). Its name is derived from *acetum*, which has meaning vinegar. It has a unique sour taste and pungent smell.

It is widely used as a food additive (code E260), mainly in the vinegar production industry. Usually, 8–10% AA is added to vinegar. AA is used to pick raw vegetables as a descaling agent to remove impurities and stains from the metal surface. It possesses excellent antibacterial property, so used as an antiseptic at a lower concentration of 1% against Pseudomonas, Staphylococci, Enterococci, Streptococci, and others, inhibiting bacterial growth by preventing biofilm formation. The chemical industry has more comprehensive application such as reagent to produce several chemical compounds such as Vinyl Acetate Monomer (VAM), an essential construction commodity as paints and adhesives, acetic anhydride, and ester. It is also used as a solvent for the recrystallization and downstream processing of various metabolites from the fermentation broth, in the production of cellulose acetate for photographic film; polyethene terephthalate for soft drink bottles, synthetic fibers, and fabrics; as well as polyvinyl acetate for wood glue (Deppenmeier et al. 2002).

Both chemical and biological methods can produce AA. Most of the growing need is fulfilled by chemical production, mainly the Monsanto process. However, chemical methods are not sustainable as it negatively impacts the environment and creates safety issues. On the contrary, the biological route has not been utilized more commercially till now. AA produced by biological means contains less AA, known as "vinegar," suitable for human consumption. An urgent demand is to develop a sustainable, cost-effective, and eco-friendly fermentative approach for AA's commercial production.

14.4.1 Biosynthetic Pathways for AA Production

Various chemical and biological methods are adopted for the commercial production of AA. The carbonylation process, also known as the Monsanto process, is a dominant process employed for AA synthesis at an industrial scale, which accounts for more than 65% of the global capacity. In this method, methanol and carbon monoxide are mixed in the rhodium-based catalyst in the liquid phase. The suitable temperature for this reaction is 150–200 °C and 30–50 bar pressure (Maitlis et al. 1996). The acetaldehyde oxidation method is another method used for the synthesis of AA in which acetaldehyde is oxidized to AA in the presence of cobalt- and chromium-based catalyst at 150 °C and 55–60 bar pressure (Sano et al. 1999). Hydrocarbon oxidation method was also developed in which butane and naphtha, hydrocarbons derived from petroleum, are oxidized using cobalt acetate and chromium acetate catalyst at a higher temperature range of approximately 150–230 °C and 50–60 bar pressure (Deshmukh and Manyar 2020). However, all these chemical methods have some serious drawbacks, such as by-products formation along with the AA such as acetone, formic acid, and propionic acid in the hydrocarbon oxidation method. Separation of by-products requires a lot of effort to remove volatile acids, and ultimately process fails to give a pure form of AA. Similarly, a yield of AA is very low in the acetaldehyde oxidation process, indicating the process is not

economically viable. In the carbonylation process, water separation is a more energy-intensive process than by-product removal to get a pure form of AA.

The biological route for AA production accounts for approximately 10% of the worldwide production and demand. Bioproduction of AA can be done under both aerobic conditions using AA bacteria and anaerobic conditions by acetogenic bacteria.

14.4.1.1 Aerobic Process

Oxidative fermentation using AA bacteria (AAB) is commercially utilized for the generation of food-grade AA, i.e., vinegar. It involves two steps: in the first step, fermentation of substrate is occurred using yeast followed by the AA bacteria (Deshmukh and Manyar 2020). Firstly, glucose is converted into ethanol using yeast, and in the next step, AAB oxidizes ethanol to produce AA. Two membrane-bound pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase and acetaldehyde dehydrogenase favor the oxidation of ethanol into acetate in two consecutive steps, and nicotinamide adenine dinucleotide (phosphate) NAD(P), located in the cytoplasm, acts as a cofactor. Alcohol dehydrogenase catalyzes the oxidation of ethanol into acetaldehyde, followed by further oxidation to AA by aldehyde dehydrogenase. Ethanol oxidation is the characteristic of almost all AAB except *Asasia*. Acetic production occurs in the following two steps.

$$\text{Glucose} \rightarrow 2\ \text{Ethanol} + 2\ CO_2 \tag{14.1}$$

$$2\ \text{Ethanol} + O_2 \rightarrow 2\ \text{Acetaldehyde} \rightarrow 2\ \text{Acetic acid} \tag{14.2}$$

14.4.1.2 Anaerobic Process

AA can be produced by acetogenic bacteria under anaerobic conditions by the breakdown of 1 mole of glucose into three moles of AA. *Acetobacterium* and *Clostridium* are well-known acetogenic bacteria used for anaerobic AA fermentation. Acetogens are well-known for their CO_2-fixing properties in which CO_2 is reduced to acetyl moiety of acetyl-CoA. In this fermentation, 1 mole of glucose is converted into 2 moles of pyruvate via glycolysis, which is further oxidized and decarboxylated to produce 2 moles of acetyl-CoA and 2 moles of CO_2 (Schaechter M 2009). These 2 moles of acetyl-CoA are converted into 2 moles of acetate. Additional acetate is generated with the fixation and reduction of CO_2 into acetate.

$$\text{Glucose} \rightarrow 2\ \text{Pyruvate} \rightarrow 2\ \text{Acetyl} - \text{CoA} + 2\ CO_2 \rightarrow 2\ \text{Acetic acid} \tag{14.3}$$

$$2CO_2 \rightarrow \text{Acetyl} - \text{CoA} \rightarrow \text{Acetic acid} \tag{14.4}$$

Aerobic fermentation resulted in 2 moles of AA, thus showed low product yield than anaerobic fermentation (3 moles AA). Substrate carbon is completely recovered (100% conversion) in the form of product in the anaerobic fermentation. Besides the higher yield in anaerobic fermentation, commercialization of the process has not been in much use due to few challenges, such as acetogenic bacteria showing

inhibitory response at higher concentration of acetate, and cannot grow effectively in acidic conditions.

14.4.2 AA Production Strategies

14.4.2.1 AA-Producing Microorganisms

Acetobacter and *Gluconobacter*, gram-negative bacteria, are the two main genera of AAB among ten classified genera used for AA commercial production in the neutral and acidic range under aerobic conditions. AAB is classified into almost ten genera, such as *Kozakia*, *Acetobacter*, *Gluconobacter*, *Asasia*, *Saccharibacter*, *Neosaia*, and *Granulibater*. These AABs are strictly aerobic, such as alphaproteobacteria, which could partially convert carbon sources to a corresponding organic compound, such as ethanol to AA. The main microorganisms reported for vinegar production are as follows: *Acetobacter aceti*, *A. oeni*, *A. cerevisiae*, *A. malorum*, *A. pomorum*, *A. pasteurianus*, *Gluconacetobacter entanii*, *G. oxydans*, *G. liquefaciens*, *Komagataeibacter europaeus*, *K. medellinensis*, *K. hansenii*, *K. oboediens*, *K. intermedius*, *and K. xylinus*. These genera are mostly utilized because of its high capacity for the oxidation of ethanol to AA and high resistance to AA released into the fermentation medium (Gomes et al. 2018). *A. pasteurianus* was reported for the commercial production of lower concentration vinegar around 6% (v/v). However, *G. europaeus* was employed to generate high-concentration vinegar (10% v/v) (Deshmukh and Manyar 2020). *Pseudomonas* sp. *CSJ-3* was isolated from the sludge and identified as a potential AA producer with glucose and ethanol (7% v/v) as substrate (Wang et al. 2018). *A. oxydans* and *Clostridium thermoaceticum* can be used for AA production at an industrial scale under aerobic and anaerobic conditions by using corn-based substrates (Busche 1991). *Saccharomyces cerevisiae* ITBR58 and *A. aceti* ITBB24 were grown on apple fermentation media and used for AA production using submerged fermentation (Rosada 2018). For industrial production, *S. cerevisiae* is mostly utilized for alcohol production than *Zymomonas mobilis,* as later showed reduced biomass production at low pH, thus reducing AA. Mixed culture of *Streptococcus lactis* ML1 and *CIostridium formicoaceticum* ATCC 27076 was used to produce AA using whey lactose as a substrate under anaerobic conditions (Tang et al. 1988).

14.4.2.2 AA Production by Various Substrates

AA can be produced from various substrates, such as pure glucose, lactose, sugarcane molasses, starch or starch-based substrates, hydrolyzed starch, grains, whey, whey lactose, malt, different fruits like apples, pears, grapes, oranges, honey, and syrup cereals. Mostly, the economic aspects are considered before any metabolite production, so the focus is mainly on the utilization of renewable carbon sources for cost-effective and efficient AA production (Deshmukh and Manyar 2020). A pure and specific substrate as well as waste substrates both can be employed for the industrial production of AA.

Specific Substrates for AA Production One of the critical steps for vinegar production is the utilization of substrate in an efficient manner to produce a higher yield of AA. Fermentable sugars and juices can be acidified under specific cultural conditions. Fruits and juices require less processing time as rich in water content, perishable. However, starchy materials, grains, and waste materials require a lot of processing before fermentation. Fruits and juices are more prone to contamination, so it requires safe handling practices. Various types of vinegar can be produced by different substrates like grapes are used to produce balsamic vinegar and apples to produce cider vinegar. Manalagi apple was reported to produce AA using *S. cerevisiae* ITBR58 and *A. aceti* ITBB24 (Rosada 2019). Grains or grains mash like barley were reported to produce malt vinegar. Mixed culture of *S. cerevisiae* and *A. pasterurianus* was reported producing AA from pure glucose. *Kluyveromyces fragilis* and *Acetobacter pasteurianus* mixed culture were reported to produce AA using whey lactose to produce AA (Parrondo et al. 2003). Whey lactose can also be used as a substrate to produce AA using a mixed culture. Lactose is converted into LA by homolactic bacteria followed by conversion into AA by homoacetic bacteria.

Economical Substrates for AA Production Waste-based substrates have gained wide attention due to the utilization of cheaper raw material to produce organic metabolites. Food waste was investigated to produce AA in a study, and one of the important advantages of using food waste is to increase environmental safety and to reduce the pollution burden (Li et al. 2015). Corn syrup was reported to use as a substrate for the formation of AA by thermophilic organism, *Clostridium thermoaceticum* under anaerobic conditions (Busche 1991). Soybean molasses, a by-product of the industry which prepares protein concentrate from soybean meal, is either discarded or used as animal feed. It contains sugars, mainly sucrose, raffinose, and stachyose, which produced ethanol, followed by AA (Miranda et al. 2020). Onion waste could be a potential source for AA production, as it contains fermentable sugars, fibers, and several bioactive compounds. In one study, simultaneous saccharification and co-fermentation (SSCF) and simultaneous saccharification and two-step fermentation processes were adopted for the AA production using *S. cerevisiae* and *A. aceti* (Kim et al. 2019). Ultrafiltered ethanol silage, corn steep liquor, and hydrolyzed soy flour were better substrates for AA production, as the increase in microbial growth and acetate production was reported (Witjitra et al. 1996). Hydrolysate obtained after pretreatment of lignocellulosic biomass wheat straw, switchgrass, forest residues, and sugarcane straw has also been used as a substrate for AA production using *Moorella thermoacetica* ATCC 39073. Pretreatment of lignocellulosic biomass resulted in fermentable sugars, such as glucose, xylose, arabinose, galactose, and mannose (Ehsanipour et al. 2016). Xylose was the first sugar consumed, followed by glucose, and then the rest of the sugars metabolized. The highest yield of AA was found in sugarcane straw hydrolysate (71%), and the lowest was recorded with forest residue (39%). Few of the studies reported for AA production from waste substrates are mentioned in Table 14.3.

Table 14.3 AA production from economical substrates

Substrate	Experimental capacity	Cultural Conditions	Microorganism	Yield	Reference
Soybean molasses	6 L	30 ± 0.5 °C	Mixed culture	40 g/L/ton	Miranda et al. (2020)
Onion waste	100 mL	30 °C	*S. cerevisiae* (KCCM 11293), *A. aceti* (KCCM 40229)	14.9–19.3 g/L	Kim et al. (2019)
Manalagi apple	250 mL	30 °C	*S. cerevisiae* ITBR58 and *A. aceti* ITBB24	6% v/v	Rosada (2019)
Food waste	500 mL	37 ± 1 °C	Yeast, acetic acid bacteria, mixed culture of yeast + AAB	12.81–25.99 g/L	Li et al. (2015)
Whey lactose	3 L	35 °C	Mixed culture of *Streptococcus lactis* and *Clostridium formicoaceticum*	20 g/L	Tang et al. (1988)

14.4.3 AA Production Enhancement Strategies

14.4.3.1 AA Production Enhancement by Strain Improvement

Bioprocess economics analysis is the most important aspect while targeting metabolite production at an industrial scale to get the maximum yield and productivity with a simultaneous reduction in overall process cost. Natural producers generally possess the limited metabolic ability to synthesize a metabolite, thus generating a low yield of products. The limited metabolic ability of natural producers creates a heavy impact on the overall cost analysis. For ages, the focus has been shifted to the strain improvement in which microorganisms are manipulated to improve the metabolic capabilities of the microorganisms up to a maximum extent. Mutation, recombination, and recombinant DNA technology are the most employed methods for strain improvement. A wild-type industrial strain *A. pasteurianus* CICIM B7003 was adopted under UV radiation with acidic stress to enhance the AA production. A mutant *A. pasteurianus* CICIM B7003-02 was obtained, exhibiting an improved yield 49.2% higher than the wild-type strain (Qi et al. 2014). This result demonstrated that other techniques, such as mutagenesis, homologous recombination, and spheroplast fusion, might lead to improved production.

14.4.3.2 AA Production Enhancement by Metabolic Engineering

Metabolic engineering can also be incorporated to enhance the yield of the desired metabolite in which the purposeful modification of cellular networks is carried out.

Modification can be done in the cellular metabolic pathways, gene regulatory networks, and signaling networks to achieve the desired yield of the metabolite. Metabolic flux analysis should be carried out carefully for a particular microorganism before proceeding toward the objective. Pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH), an enzyme involved in the ethanol oxidation reaction, was investigated in AA bacteria *Acetobacter pasteurianus* JST-S. Two subunits of the PQQ-ADH enzyme (adhA) and (adhB) were overexpressed using *E. coli* plasmid, and its effect on AA production was studied. The total AA content was enhanced to 61.42 g/L in an engineered strain higher than the original strain (52.23 g/L) (Wu et al. 2017). Similarly, other metabolic pathways and related enzymes can be studied, and yield can be maximized.

Another method to improve the metabolite yield is the genetic manipulation of the microorganism in which a gene of interest can be manipulated to achieve the desired goal. One of the crucial aspects of AA bacteria's fermentation is the resistance to AA and ethanol concentration. Genes responsible for AA resistance can be identified and manipulated to enhance the yield of AA. In a study, overproduction of aconitase protein was reported at a higher concentration of AA in *Acetobacter aceti*. Further, the cloning of the genes encoding protein was done to check the effect on microbial growth and AA yield. Overexpression of the aconitase gene leads to a higher yield of AA and reduced fermentation time (Nakano et al. 2004). This result suggests that further cloning of AA and ethanol resistance genes can be altered to induce AA yield and productivity.

14.4.3.3 AA Production Enhancement by Reactor Selection and Mode of Fermentation

AA production requires two major steps: firstly, glucose is converted into ethanol by yeast under anaerobic conditions; later, alcohol gets converted into AA using AAB under aerobic conditions. Both the steps and microorganisms require different cultural conditions. Various cultural conditions, types of reactor, and mode of the process can improve the yield and productivity of AA. A few of the critical factors that can affect the fermentation process are either batch, fed-batch, or continuous mode; use of single or mixed culture; pH; temperature; and maintenance of aerobic/anaerobic conditions.

At the start of the industrial production, partially filled wine casks were inoculated and exposed to air for fermentation. Later, holes were bored on the casks, and glass tubes were inserted to remove vinegar in "Orleans method" to reduce the AA resistance. Solid-state fermentation (SSF) was also employed, in which microorganisms are grown on the surface of a substrate such as grains in the absence of free water. It involved three steps: starch liquefaction and saccharification, alcohol fermentation, and AA fermentation. It showed lower productivity as it required more fermentation time and oxygen availability was the major limitation. Surface fermentation "trickling or German/generator process" was also adopted on an industrial scale in which microorganism is attached to a support, such as a beechwood shavings, and alcohol could trickle down through the support and the air sparging was done from the bottom. Bottom content, composed of partially converted

solution, can be recycled again through the support until the reaction is complete (Schaechter 2009).

Submerged fermentation, in which liquid media is used for production, replaced the other methods later at an industrial scale. Submerged fermentation is advantageous over SSF and surface due to high yield and productivity due to faster oxidation of ethanol, smaller reaction volumes, and lower capital costs. Further, the efficiency can be enhanced by introducing strategies for better process control in terms of oxygen availability, temperature, and AA and ethanol content. The submerged semicontinuous process was the choice over the continuous process for the industrial vinegar production in which once the AA concentration reaches up to a level. The spent solution is removed and replaced with the fresh substrate (ethanol) solution. Batch fermentation mode using *Psuedomonas* sp. *CSJ-3* was adopted in a study. Fermentation media was composed of glucose as well as ethanol 7% (v/v). Optimization studies of cultural conditions were carried out, and yield was 0.49–0.59% with the optimized parameters (Wang et al. 2018). The continuous process was also employed in the bubble column reactor of 6–60 L capacity for the wine vinegar production (Fregapane et al. 2003). Cultural conditions were maintained at 30 °C temperature and airflow rate of 0.125 vvm. The concentration of AA was found to be 68.2 g/L which is higher in comparison to the semicontinuous process (45.1 g/L).

Continuous fermentation using immobilized cells has also been used to improve productivity, but oxygen transfer is the major limitation of the immobilized cells. AA is a growth-associated metabolite. Thus, retention of the biomass in the reactor is the most crucial aspect. Cell recycle bioreactors with a membrane module have been used to increase the productivity of the process. Cell recycling is better than immobilized cells due to no oxygen diffusion limitation. As the process show AA and ethanol resistance, a fed-batch mode can be preferred to improve the AA yield. Fed-batch mode is best suited for the substrate inhibition cases. Inhibition of microbial growth occurs in a higher concentration of ethanol due to a change in the media's pH. In one of the studies, the co-culture of *Lactococcus lactis* and *Clostridium formicoaceticum* was reported for AA production in a fed-batch fibrous bed reactor, eliminating the clogging and channeling problems and showed sustainable performance for a longer duration of time (Huang and Yang 1998). Chemostat cascade could also be a better alternative to enhance the AA yield and productivity, because different cultural conditions suitable for yeast and AA bacteria can be maintained in different bioreactors connected in series. The end product of the first reactor is the substrate for the next batch in the chemostat cascade.

14.4.3.4 AA Production Enhancement by Optimization

Wild-type microorganism usually produces metabolites in a meager quantity. However, this quantity can be enhanced up to a certain level based on the maximum ability of the microorganism, by manipulating and optimizing cultural conditions and media constituents. For the cost-effective commercial production of metabolites, improved strain and optimized parameters should be employed. pH, temperature, AA, and ethanol concentration of the media are the critical factors that can enhance or decline the AA concentration. Optimum pH was reported between 7.0 and 7.6 for

the mixed culture fermentation using *S. lactis* and *C. formicoaceticum*. Extraction and recovery of AA were found to be difficult if pH maintained higher than 7.6 (Tang et al. 1988). The effect of pH, temperature, initial AA, ethanol, CA, and LA concentration was monitored on AA production by few strains of AAB. The carbon source, nitrogen source, C/N ratio, minerals, and vitamin addition could significantly affect the yield and productivity according to the microorganism used. The aeration rate was also suggested to impart a significant impact on the AA yield.

14.4.4 AA Purification Strategies

Downstream processing of any metabolite accounts for more than 75% of the overall process cost. Various strategies can be incorporated to enhance the maximum recovery of AA from the fermented broth and reduce the recovery cost. Chemical synthesis of AA like methyl carbonylation method is mainly employed at an industrial level, which is a multistep and multiphase process and requires several kinds of unit operations. The chemical route is thus unable to maintain a clean and green production environment. The focus has slowly shifted toward the fermentative approaches for AA production using sustainable production strategies to reduce the environmental burden. The major challenge of fermentative production is the downstream processing of AA in a cost-effective and efficient eco-friendly way. As raw materials used for the fermentation are not of high purity, the purity of the end product is the most significant task. AA and ethanol resistance is the major problem of AA production as lower pH inhibits microbial growth. Thus, inhibition of AA production occurs as AA is growth associated metabolite. Continuous separation of AA from the fermented broth is desired to maintain microbial health, which can be achieved with the help of the membranes. The first step of the recovery and purification is biomass separation, which can be done by microfiltration (MF).

Further, AA can be best concentrated with the help of nanofiltration (NF) as well as electrodialysis. Later, evaporation of permeate/dialyzed content can be followed to get a pure form. The efficiency of a process is dependent on pressure, pH, temperature, and other media component concentration (Han and Cheryan 1996). For the more purified industrial grade, more techniques can be combined. The utilization of techniques such as chromatography, distillation, liquid-liquid extraction, precipitation, and crystallization is dependent on the various physical and chemical properties of the cell-free fermented broth. Freeze concentration is the preferred method for fermentative production (Schaechter 2009).

14.4.5 Challenges and Future Scope for AA Bioprocessing

The major drawback associated with AA industrial production is the utilization of conventional chemical methods for the worldwide growing demand for AA, impacting environmental issues. Conventional methods used for the 90% demand are the methyl carbonylation process, the Cativa process that synthesizes lots of

by-products, and the desired product. Separation of the AA from the rest of the components is quite labor-intensive and requires more energy. Water is also needed for recovery; thus, all these factors contribute to the environmental burden. So, there is a need to develop a green and sustainable approach for AA commercial production, which should be energy efficient. Nowadays, the anaerobic fermentative approach is trending with cheaper raw materials to get the pure form of AA in a cost-efficient manner. However, getting the maximum purity of AA from the impure substrate through various unit operations is the primary task of this approach. AA resistance is the main problem that inhibits microbial growth, thus hampering an efficient production process.

A membrane integrated fermentation system with operational simplicity should be designed to eliminate end-product inhibition and efficient recovery of the metabolite. A chemostat cascade reactor coupled with the membrane should be developed in which both the steps and microorganisms can be monitored efficiently to enhance AA productivity. Scale-up of the membrane-based reactor is the crucial aspect implemented before commercial production. Further, the use of mixed culture optimization according to the nutritional requirement of microorganisms can maximize AA production.

14.5 Kojic Acid (KA)

The term "kojic acid" was derived from "koji," which is that the inoculum utilized in Eastern food fermentations for several centuries (Mohamad et al. 2010). Industrially, KA was a product of aerobic fermentation using *Aspergillus* species. Chemically, KA is 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one which is an organic acid and produced biologically by many fungi in the aerobic fermentation process by different carbon sources, including arabinose, glucose, sucrose, and xylose (Chaudhary et al. 2014). In 1907, Saito discovered *Aspergillus oryzae* mycelia contain KA when grown on steamed rice. Yabuta reported the chemical structure of KA in 1924 as shown in Fig. 14.3 (Zirak and Eftekhari-Sis 2015).

KA has various commercial applications, such as in cosmetic, agriculture, medicine, food, and chemical industries. Because of its importance in various industries, the demand for KA is increasing day by day, so significant research has been dedicated to the biosynthesis of KA and its chemical and biological properties.

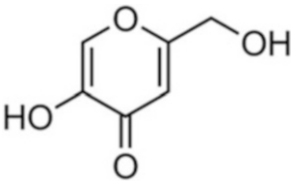

Fig. 14.3 Structure of kojic acid (Zirak and Eftekhari-Sis 2015)

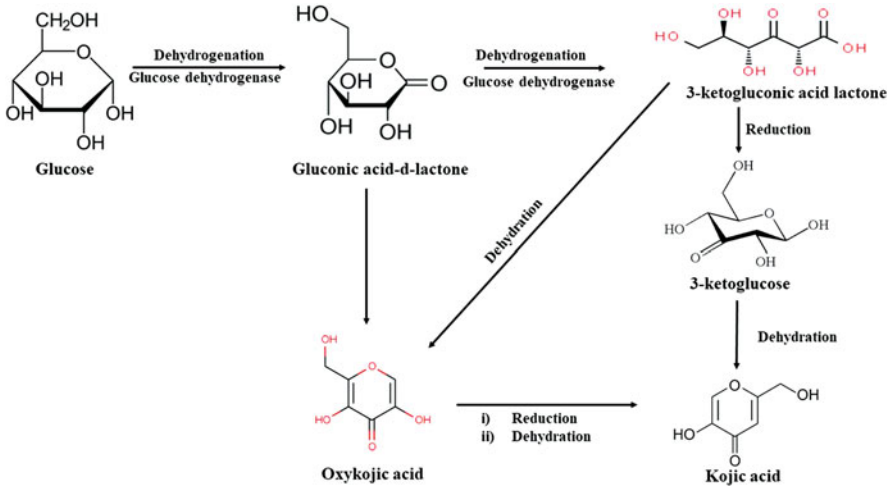

Fig. 14.4 Kojic acid biosynthesis pathway (Bajpai et al. 1981)

14.5.1 Biosynthetic Pathways for KA Production

The biosynthesis of KA from glucose follows any of the three pathways, as shown in Fig. 14.4.

1. KA formation from glucose through three-step conversion, i.e., glucose to gluconic acid-d-lactone to oxykojic acid to KA by reduction and dehydration.
2. Another pathway involving the formation of KA, which results in the dehydrogenation of gluconic acid-d-lactone following reduction of 3-ketogluconic acid glucose and dehydration of 3-ketoglucose.
3. The third pathway involving the dehydration of 3-ketogluconic acid lactone forming an intermediate oxykojic acid and final product as KA by reduction and dehydration.

The glucose dehydrogenase and gluconate dehydrogenase are the main enzymes of the biosynthetic pathway. These pathways presented support theoretical grounds incorporating gluconic acid as an intermediate. KA biosynthesis may involve three metabolic pathways as follows and shown in Fig. 14.4 (Bajpai et al. 1981).

14.5.2 KA-Producing Microorganisms

Using various carbon sources, a variety of microorganisms in an aerobic process produce KA. Commonly, many *Aspergillus* spp., belonging to the *A. tamarii* group, are being utilized in KA production. Kitada et al. (1967) and Kwak and Rhee (1992) produced 0.26 g-KA/g-glucose by employing *A. oryzae* (Kitada et al. 1967; Kwak

and Rhee 1992). Additionally, KA production by *A. parasiticus* and *A. candidus* resulted in yields of 0.089 g-KA/g-glucose and 0.3 g-KA/g-sucrose, respectively (El-Aasar 2006; Wei et al. 1991). *A. flavus* was also reported to produce a high yield (0.453 g/g glucose) (Rosfarizan and Ariff 2006). However, the chance of aflatoxin production by this strain could not be ignored. An appropriate medium formulation and culture conditions may reduce the aflatoxin production by *A. flavus*. Additionally, there is no coupling of KA and aflatoxin biosynthesis pathway; thus, aflatoxin production by *Aspergillus* sp. can be controlled (Basappa et al. 1970).

14.5.3 KA Production by Various Substrates

14.5.3.1 Specific Substrates for KA Production

Carbon source. Carbon-containing compounds (Table 14.4), such as acetate, arabinose, ethanol, fructose, galactose, glucose, glycerol, maltose, mannose, sorbitol, starch, sucrose, and xylose, could act as a substrate for the KA production (Arnstein and Bentley 1956; Rosfarizan and Ariff 2006). Glucose gave the highest yield, which ranged between 0.5 and 0.6 g of KA, followed by sucrose and fructose (Mohamad et al. 2010).

Nitrogen Source Various nitrogen sources are selected for KA production using different strains of *Aspergillus* sp. It is reported that organic nitrogen sources are superior to inorganic nitrogen sources for KA production by Kitada et al. (1967) as shown in Table 14.5. Complex nitrogen sources, including peptone and yeast extract, act as a precursor for KA production. The addition of some organic nitrogen sources can produce a buffering system, whereas inorganic nitrogen sources, such as ammonia, significantly reduce the media pH due to ammonium ion absorption. Low pH may affect the microbial KA synthesis and inhibit its growth (Rosfarizan and

Table 14.4 Carbon sources for kojic acid fermentation

Carbon source	Microorganism	Reference
Ethanol	*A. oryzae*	Basappa et al. (1970)
Glycine	*A. flavus*	Arnstein and Bentley (1956)
Sodium acetate	*A. oryzae*	Arnstein and Bentley (1956)
Glycerol	*A. tamarii*	Gould (1938)
Fructose	*A. tamarii*	Gould (1938)
Glucose	*A. luteo-virescens* *A. oryzae* *A. flavus* *A. candidus* *A. parasiticus*	Morton et al. (1945) Kitada et al. (1967) Wan et al. (2005) Wei et al. (1991) El-Aasar (2006)
Maltose	*A. oryzae*	Kitada et al. (1967)
Sucrose	*A. flavus*	Rosfarizan and Ariff (2006)
Starch	*A. flavus*	Rosfarizan and Ariff (2000)

Table 14.5 Nitrogen sources for kojic acid fermentation

Microorganism	Nitrogen source (g/L)	Reference
A. oryzae	Ammonium sulfate Yeast extract	Arnstein and Bentley (1953) Kitada et al. (1967), Ogawa et al. (1995)
A. flavus	NH_4NO_3 Yeast extract	Fadahunsi and Garuba (2012), Megalla et al. (1987), Wan et al. (2005)
A. tamarii	Sodium nitrate	Gould (1938)
A. albus	Polypeptone	Saruno et al. (1979)
A. candidus	Yeast extract	Wei et al. (1991)
A. parasiticus	Peptone Yeast extract	Coupland and Niehaus Jr (1987) El-Aasar (2006)

Ariff 2000). Yeast extract is preferred over peptone and polypeptone as an organic nitrogen source for KA production.

C/N Ratio

Optimization of carbon to nitrogen ratio (C/N ratio) is crucial as it significantly affects KA production. The critical C/N ratio for KA production was found to be 100. Below this value, KA production was found to be significantly decreased. However, a C/N ratio higher than 100 does not considerably increase the KA production (Mohamad et al. 2010). This leads to the conclusion that a nitrogen-limited environment favors KA production (Rosfarizan and Ariff 2000).

14.5.3.2 Economical Substrate for KA Production

Agriculture leftover is rich in various bioactive compounds. These residues can be utilized as a suitable source for the KA production, and thus it helps to reduce the production cost and reduce pollution, saving the environment. Agro-industrial wastes, including fruits, vegetables, agriculture by-products, pickle wastes, corn steep liquor, black-strap molasses, and cheese whey, are used in solid-state fermentation (SSF) (Veana et al. 2014). A variety of microorganisms are used to produce these valuable products through SSF processes (Krishna 2005). These widely available raw materials can also be used for KA production, which will also recycle the by-product of agriculture and industry.

14.5.4 KA Production Enhancement Strategies

14.5.4.1 KA Production Enhancement by Strain Improvement

KA producer strains, such as *A. flavus*, have been developed for the higher amount of KA production (up to 19.2 g/L), using monospore isolation technique (Rosfarizan et al. 1998). Mutation and genetic recombination techniques can be applied to improve KA-producing strains (Crueger and Crueger 1990). KA production by mutation of a phototropic fungi *A. oryzae* (green conidia) has been reported,

which produces KA five times above the parent strain (Demain 1981). A mutated strain of *A. oryzae* MK107-39 has also produced about 7.7 times more KA, than its parent strain (Futamura et al. 2001). *A. oryzae* ATCC 22788 was mutated by NTG treatment, UV irradiation, which was followed by protoplast fusion, which led to about 100 times higher production (41 g/L) (Wan et al. 2004).

14.5.4.2 KA Production Enhancement by Mutagenesis

Mutation has been a valuable tool for many decades to develop strains for enhanced production of metabolites having commercial importance. KA yield has been improvised by inducing mutations in two *Aspergillus* strains, namely, *A. oryzae* HAk2 and *A. flavus* HAk1, through gamma radiations by 2.03- and 1.9-fold, respectively. The results obtained indicated the positive influence of gamma irradiation on KA production by a mutation in the responsible gene clusters (Ammar et al. 2017). A similar result was obtained by Abd El-Aziz (2013), when the parent strain of *A. flavus* AFNS9 was mutated, by using gamma radiation (Abd El-Aziz 2013). In another work, a novel strategy of combined mutagenesis was used for the enhancement of KA production in an isolated strain of *Aspergillus oryzae*. The methods of mutagenesis included microwave irradiation, heat-LiCl, UV irradiation, heat-LiCl, atmospheric, and room temperature plasma (ARTP). The result showed an increase in KA production which ranged from 47% to 292.3%, which was a quite promising enhancement (Feng et al. 2019).

14.5.4.3 KA Production Enhancement by a Selection of Reactor and Mode of Fermentation

The selection of bioreactor plays an important role in the determination of product yield. Submerged fermentation carried out in batch mode resulted in maximum yield, KA concentration, and productivity (Mohamad et al. 2010). A study was carried out to estimate the effect of airlift reactors on KA production using corn steep liquor with hydrolyzed corn starch. The result showed that the cost of energy requirement was reduced to one-fourth of the cost required in a jar fermenter (Futamura et al. 2001). In another study, a bioreactor was developed for KA production by *A. oryzae on* a plastic composite support in a nitrogen-limited condition. This design allowed repeated batch fermentation, which led to enhancement in productivity to 3.09 g/L/d. Thus, bioreactors supporting immobilization technique have the potential for scale-up to industrial scale (Liu et al. 2016).

14.5.4.4 Specific Precursors for KA Production

In KA biosynthesis, the two monosaccharides act simultaneously: Glucose acts as a precursor of KA, and fructose contributes to microbial cell growth (Hassan Saeed 2013). In another study of a precursor of KA formation, it was demonstrated that yeast extract is a complex nitrogen source containing vitamins (Shakibaie et al. 2018).

14.5.4.5 KA Production Enhancement by Optimization

Optimization is improving the performance of a process or a product to obtain the maximum benefit from it. In the last decades, the different mathematical tools, RSM and ANN, have been applied for optimization and process modeling (Elfghi 2016).

Various reaction conditions in KA synthesis, such as substrate ratio, amount of enzyme, reaction temperature, reaction time, and solvent polarity, have been studied to understand the process better. A statistical method such as RSM has gained hugely by researchers to investigate their operating conditions and interactive relationships due to its advantages of reducing the number of experimental runs, which is sufficient to provide a statistically acceptable result (Lajis 2016).

14.5.5 KA Purification Strategies

Repeated crystallization is one of the preferred methods for KA downstream processing, which results in impurities-free KA crystals. The crystallization process consists of the following steps: (1) KA solution is prepared from fermentation broth by using suitable solvent. (2) Additional KA extraction is performed using suitable solvents such that the final concentration is 80 g/L–100 g/L. (3) The concentrated solution is agitated at 50–100 rpm. (4) the KA solution is then concentrated by evaporation of the solvents at 50 °C. (5) Concentrated KA solution is then maintained at 5 °C to 35 °C for about 10 h to obtain crystals of KA. (6) KA crystals are then separated. (7) Steps from (2) to (6) are then repeated to obtain high purity KA crystals (Ishak et al. 2016).

14.5.6 Challenges and Future Scope for KA Bioprocessing

The large demand and use of KA in various industries, such as food (as antioxidant, discoloration agent, and flavor enhancers), cosmetic (as skin lightening agent), pharmaceutical (as a painkiller, anti-inflammatory agent, and bacterial inhibitor), and agriculture (prevents melanosis), have led to a challenge of boosting investigation to enhance KA production at large scale (Chaudhary et al. 2014; El-Kady et al. 2014; Saeedi et al. 2019). The global KA market size was US$ 35 million in 2019, which is expected to rise to US$ 39 million. The expected CAGR for the KA market is 1.4% during 2021–2026 (Mahesh et al. 2019).

14.6 Itaconic Acid (IA)

Itaconic acid (IA), a white crystalline compound, is also known as 2-methylidenebutanedioic acid, is a platform chemical with widespread uses. The applications of this organic acid are in the areas of food, pharmaceutical, polymer, water treatment, detergent, and agriculture. According to a report published by the US Department of Energy in 2004, IA holds a place in the top 12 building blocks that

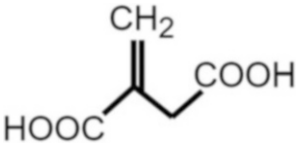

Fig. 14.5 Chemical structure of Itaconic acid

can be converted into high-value chemicals or materials (Werpy and Petersen 2004). As a building block of polymers, this organic acid has been explored for many decades. This application is attributed to the chemical structure of IA, which contains one unsaturated bond and two carboxylic acid groups, as shown in Fig. 14.5. This structure enables this organic acid to polymerize with itself as well as acrylonitrile. Thus, IA can act as a precursor of methyl methacrylate (a bio-based thermoplastic material) and a potential biofuel known as 3-methyl tetrahydrofuran (Yang et al. 2019). IA has been found to be highly soluble in water and methanol. Organic solvents, such as 2-propanol, ethanol, and 1-propanol, can also dissolve IA with solubility in decreasing order (da Cruz et al. 2017).

Kinoshita et al., in 1931, investigated the biochemical production of IA from a species named *Aspergillus itaconicus,* which was grown on salted plum juice (Kinoshita 1931). Table 14.6 summarizes the historical developments in the production of IA in chronological order.

The chemical route for IA production has also been studied. This includes aconitic acid decarboxylation, isoprene oxidation, acetylene derivative carboxylation, and CA distillation, followed by anhydride hydrolysis (da Cruz et al. 2017). However, the economic feasibility of chemical synthesis is low due to a large number of steps and low yield.

14.6.1 Biosynthetic Pathways for IA Production

Several investigations were carried out for understanding the mechanism underlying the biosynthesis of IA. According to Kinoshita, *A. itaconicus* synthesize IA by decarboxylation of aconitate (Kinoshita 1931). Based on this understanding, investigations were carried out for the intermediate role of citrate in the biosynthesis of IA (Klement and Büchs 2013). Klement et al. described an important milestone achieved, by Bentley et al., which was to decipher the role of cis-aconitate decarboxylase (CAD) in catalyzing the conversion of cis-aconitate to IA (Klement and Büchs 2013). Several studies were carried out to demonstrate the role of CAD and aconitase enzyme in the biosynthesis of IA (Dwiarti et al. 2002; Huang et al. 2014; Kanamasa et al. 2008; Tevž et al. 2010; van de Kant et al. 2010; Yang et al. 2017).

The pathway studied in *A. terreus* includes utilization of glucose as substrate and its oxidation by Embden-Meyerhof-Parnas (EMP) pathway in the cytosol, leading to the formation of two molecules of pyruvate. One of the pyruvate molecules so formed is carried to the mitochondria by a transport protein known as pyruvate translocase. Pyruvate is then converted into acetyl-CoA, which reacts with oxaloacetate in the tricarboxylic acid (TCA) cycle to form cis-aconitate with the enzyme

Table 14.6 Historical development in the production of IA

S. no.	Historical milestones in the biological production of IA	Reference
1.	IA is a metabolic product of *Aspergillus terreus* with 12% yield	Calam et al. (1939)
2.	The first patent for commercial production of IA using microbial route	Kane et al. (1945)
3.	*A. terreus* NRRL 1960 was isolated as IA-producing strains by some scientists working at NRRl, USA. This strain yielded 30% production of IA at a semi-pilot scale, due which it was established as the most widely used strain	Lockwood and Ward (1945)
4.	Production of IA at pilot plant scale with NRRL 1960 by submerged fermentation initiated	Pfeifer et al. (1952)
5.	Role of pH on IA production by *A. terreus*	Larsen and Eimhjellen (1955)
6.	Possible role of cis-aconitate decarboxylase in the biosynthesis of IA	Bentley and Thiessen (1957a), Bentley and Thiessen (1957b), Bentley and Thiessen (1957c)
7.	The improved production process was patented, which reported an increase in yield of IA up to 50% by use of cane and beet molasses	Nubel and Ratajak (1962)
8.	Growth and oxygen requirements of *A. terreus* for IA production	Gyamerah (1995)
9.	Study of a metabolic pathway for IA production in *A. terreus*	Bonnarme et al. (1995), Jaklitsch et al. (1991)
10.	Isolation and characterization of cis-aconitate decarboxylase enzyme from *A. terreus*	Dwiarti et al. (2002)

aconitase. Cis-aconitate is then transported to the cytosol by a mitochondrial carrier family protein, where enzymatic catalysis by CAD leads to the synthesis of itaconate. The second pyruvate molecule so formed converts into oxaloacetate by combining with one molecule of CO_2 in the cytosol in an anaplerotic reaction. This oxaloacetate is either carried directly to the mitochondria or is converted into malate, which is then carried inside the mitochondria. In either case, the carriers involved are mitochondrial tricarboxylic acid transporters (MTT). Studies suggest that malate, fumarate, and oxaloacetate are preferable precursors for IA synthesis, and citrate is not a good precursor for IA synthesis. This is also supported by the fact that *A. niger* is a significant producer of CA and does not exhibit generous yields of IA (Klement and Büchs 2013). The detailed biosynthetic pathway in two natural producers *Aspergillus terreus and Ustilago maydis* has been described in other literature (Tehrani et al. 2019; Wierckx et al. 2020).

14.6.2 IA Production Strategies

The production of IA needs to be understood from a biosynthetic perspective and its regulation in the producer organisms. The common producer of IA, *A. terreus* has pyruvate carboxylase (PC) located in the cytoplasm, which helps in the utilization of the CO_2 molecule generated. Similarly, other microorganisms have dedicated pathways for IA biosynthesis. The TCA cycle enzymes have been associated with the downregulation of IA production. Similarly, high-energy load decreases IA production, as evidenced by downregulation caused by a high concentration of ATP and NADH (Klement and Büchs 2013). These environmental conditions must be supported by the presence of CAD enzymes. Another important characteristic of high IA producers is the presence of a dedicated transporter system to transfer TCA cycle intermediates, such as cis-aconitate to the cytoplasm. These conditions can facilitate the production of IA in any microorganism.

14.6.2.1 IA-Producing Microorganisms

According to Klement et al., some major producers of IA include *A. itaconicus*, *A. terreus*, *Ustilago maydis*, *Candida* sp., and *Pseudozyma antarctica*. However, *A. terreus* is the highest producer of IA under optimized conditions (Kuenz et al. 2012). The production of other nonfilamentous microorganisms can also be enhanced by lowering the operating pH and reducing other by-products, such as malate and hydrocarbons. Many studies have been carried out to express the CAD genes in recombinant strains listed in Table 14.7.

The screening of producer microorganisms also depends on cultivation conditions. These conditions should meet the requirements for enhanced IA production. Several substrates have been evaluated for their effect on IA production, which has been discussed in the following section.

14.6.2.2 IA Production by Various Substrates

Substrate selection has a critical role in the determination of the overall cost of the product. The yield obtained with a particular substrate must be analyzed in terms of production cost. Thus, this factor has two components; one is the yield, and the second is the cost and availability of the substrate. Some costly substrates may have higher yields as compared to the widely available low-cost substrate. Also, the complexity of the substrate degradation and pretreatment may impose bottlenecks

Table 14.7 List of studies conducted to express CAD genes in recombinant strains

Microorganism	Modifications	Reference
E. coli pET-9971	CAD enzyme	Li et al. (2011)
E. coli BW25133	CAD enzyme	Liao and Chang (2012)
Aspergillus niger AB 1.13	CAD enzyme	Calam et al. (1939)
Yarrowia lipolytica	CAD enzyme, citrate synthase, and aconitase	Wang et al. (2012)

during downstream processing. Thus, the selection of the substrate should be made in a rational manner.

Levinson et al. evaluated the effect of the addition of different carbon sources on IA production by *Pseudozyma antarctica* NRRL Y-7808 in a nitrogen-limited condition. This study carried out in a stirred tank reactor showed that the maximum IA was produced in the presence of glucose and fructose. Sucrose and maltose were less efficiently utilized. Glycerol and maltose showed the minimum IA production among all the evaluated carbon sources (Levinson et al. 2006).

Saha et al. evaluated the production of IA by six different *Aspergillus terreus* strains in a 96-well microtiter plate using different substrates, such as glucose, xylose, arabinose, and their combinations (Chang et al. 2017).

Economical Substrate

As the IA producer organisms such as *A. terreus* have the ability to utilize hemicelluloses, so a variety of lignocellulose-based agricultural residues have the potential for IA conversion (Klement et al. 2012; Kobayashi 1978). This concept can lead to the incorporation of IA production in a circular economy by combining them with biorefineries. However, some of the toxic compounds present in hardwood were found to inhibit IA production. Also, it has been suggested that the microorganism must be efficient in the utilization of both cellulose and hemicelluloses. This is associated with a low yield of IA with xylose (Klement and Büchs 2013). Klement et al. also proposed that substrates such as corn starch should be preferred over potato starch due to the high concentration of phosphorus in the latter, which can decrease the yield of IA production. IA production has been quite sensitive to the presence of inhibitors; therefore, the selection of any plant material as substrate involves detailed characterization of phytoconstituents and assessing their potential to act as an inhibitor. This hurdle can be removed by the use of some pretreatment strategy, which may be limited by the increased cost of IA production.

Other alternative raw materials evaluated for IA production by *Aspergillus niveus* include wheat flour, corn starch, and sweet potato (Gnanasekaran et al. 2018). However, the phosphorus content of sweet potatoes may inhibit IA production, as discussed previously.

14.6.3 IA Production Enhancement Strategies

Many of the process parameters, such as pH, oxygen supply, and nutrient limitations, have been reported to affect IA production. Oxygen supply is one of the primary factors on which IA production depends; however, meeting this requirement becomes complex due to the shear damage created at a high stirring rate of the impeller. According to a finding, an increase of oxygen concentration in the supplied air can significantly enhance the production of IA without damaging the fungal mycelium at high impeller tip speed (Park et al. 1993). The effect of DO concentration and impeller tip speed on IA production by *A. terreus* has been studied. It has

been estimated that for the synthesis of 1 mol of IA, 1.5 mol of oxygen is required (Yang et al. 2019).

Other than oxygen requirement, pH level also plays a critical role in IA production. A low pH value not only provides an auto-sterile environment but also increases IA production significantly. However, this pH cannot be lowered below a specific value (pH 2.0) as both IA production and cell morphology are adversely affected. The shake flask culture also shows a significant level of IA when the initial pH is maintained at a low value of about pH 3.0. Other than phosphate, lower ammonium concentration also favors IA production by NADH regeneration. The addition of calcium has been recommended for high IA production as it inhibits the conversion of IA to itatartaric acid and acts as a cofactor of enzymes, such as PEP carboxylase (Gyamerah 1995).

14.6.3.1 IA Production Enhancement by Metabolic Engineering

A major breakthrough in pathway engineering to enhance IA production was carried out by introducing the Vitreoscilla hemoglobin gene in the mutant strain of *A. terreus,* which led to a 40% enhancement in IA production in the shake flask study (Shin et al. 2009). This enhancement was possible due to the removal of oxygen limitation, which is a major factor of IA production. Several genes such as *cadA* and *mfsA* have been overexpressed to enhance IA production in *A. terreus* (Huang et al. 2014). Similarly, IA biosynthesis was expressed in *A. niger* by the overexpression of CAD, MTT, and MFS genes, leading to 7.1 g/L of IA production (Liu et al. 2017). In another study, *Pichia kudriavzevii* was used as a host and engineered to produce IA, which was based on pH-tolerant characteristics of this yeast. Many attempts have been made to express IA synthesis in alternative hosts, such as *Saccharomyces cerevisiae*, A*spergillus niger*, *Yarrowia lipolytica*, *Escherichia coli*, and *Corynebacterium glutamicum.* However, the production titer of IA in *A. terreus* is still the highest (Sun et al. 2020).

14.6.3.2 IA Production Enhancement by Reactor Selection and Mode of Fermentation

Some early IA production studies were carried out using airlift reactor (Lyngstad and Grasdalen 1993; Okabe et al. 1993; Park et al. 1994; Yahiro et al. 1995). Another study evaluated the effect of immobilization of a strain of *A. terreus* on polyurethane foam in a column bioreactor, operated in continuous mode for 4.5 months (Kautola et al. 1990). Carstensen et al. in 2018 published a study in which IA was produced using a membrane bioreactor to integrate the production and primary purification steps. This strategy involved "reverse flow diafiltration," which reduced the oxygen limitations and the hydromechanical stress (Carstensen et al. 2013). To de-bottleneck the problem of oxygen limitation, a study was recently conducted where 0.31 g-IA/g-glucose and 3.15 g-IA/L/day were obtained with an oxygen inlet concentration of 31.5% (v/v). The yield and productivity were found to be 0.32 g-IA/g-glucose and 4.26 g-IA/L/day, respectively, when the agitation rate was 600 rpm. This study was conducted using a stirred tank bioreactor with 1.5 L as working volume (Nemestóthy et al. 2019).

14.6.3.3 IA Production Enhancement by Optimization

The scope of optimization of IA production lies in increasing productivity as a significant lag phase is observed when the fermentation is carried out with a fungal strain such as *A. terreus*. This can also be achieved by transferring the responsible genes in a suitable host having a shorter doubling time. A high concentration of IA is also a desirable phenomenon that can be achieved by overcoming product inhibition. Recombinant DNA technology can be applied to obtain hyperproducer strains resistant to product inhibition (Yahiro et al. 1995). Optimization studies have also been carried out to evaluate the effect of different media components on IA production. One such study was conducted using a full factorial design, which showed that carbon to nitrogen ratio (C/N ratio) is one of the major factors affecting IA production (Jang et al. 2009). Similarly, Bafana et al. evaluated the effect of factors, such as salts, peptone, pH, and inoculum conditions, on IA production by *A. terreus*. This optimization study led to a 143-fold increase in production using deionized potato waste, which can be correlated to phosphate removal (Bafana et al. 2019).

14.6.4 IA Purification Strategies

Purification of organic acids involves 30–40% of the overall cost of production. Purification strategy includes selective removal of IA from the broth by using unit operations, such as adsorption, electrodialysis, membrane separation, and crystallization. The process of crystallization is cost-intensive due to the large energy requirement during the evaporation process. It has also been found that a high concentration of protonated IA causes a reduction in productivity due to its product inhibition effect. To solve this bottleneck, an in situ product removal strategy has been suggested by Eggert et al. (Eggert et al. 2019). Another study based on reactive extraction was conducted by Kreyenschulte et al., in which they used trioctylamine and isopropyl myristate as the extraction mixture (Kreyenschulte et al. 2018). Techno-economic feasibility analysis of IA purification was conducted using different purification strategies. The cost involved in upstream processing and fermentation was calculated as about 1.13 US$Kg^{-1}, whereas the cost of purification using the crystallization strategy was calculated as 0.89 US$Kg^{1}. This study suggested that this cost of crystallization can be reduced to 0.63 US$Kg^{-1}, by replacing crystallization with adsorption as the primary downstream processing method (Magalhães Jr et al. 2019).

14.6.5 Challenges and Future Scope for IA Bioprocessing

As IA finds several industrial applications, its production enhancement needs further detailed insight. One major challenge associated with IA production is high oxygen requirement. This oxygen requirement can be met by high oxygen concentration in the inlet gas and high agitation rate, as observed by a study (Nemestóthy et al. 2019). However, a high agitation rate is also limited by the shear damage caused to the

fungal mycelia. Thus, the effect of increased agitation rate on fungal morphology needs to be further examined. Attempts have been made to develop unicellular recombinant host strains for IA production; however, the yield has been compromised as compared to *A. terreus*. Thus, the development of a novel strain with comparable IA yield, which can be scaled-up for industrial bioreactors, utilizes agro-waste as substrates, leading to a significant reduction in production cost that can be integrated into circular biorefinery. The production strategy with negligible product inhibition should be coupled to an energy-efficient purification strategy. This objective needs further investigations to improve IA production at a commercial scale.

14.7 Conclusion

Based on the analysis carried out in this chapter for microbial production of organic acids, a strategic approach for each organic acid must be designed. The production method adopted at lab scale for optimization purposes must be scalable to the industrial level. The bottlenecks in each process can be resolved by analysis of the metabolic pathways and engineering them. Techno-economic analysis of these products should be done, and more attempts are required for integrating them in a circular economy. These low-value high-volume products have high market demand. Thus, the future lies in further investigations of process development for enhanced productivity.

Declaration of Competing Interest The authors declare that they have no competing interest.

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Biological Production of Succinic Acid: State of the Art and Future Perspectives

15

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Abstract

Succinic acid has been extensively used as a precursor for the synthesis of various pharmaceutical- and food-grade chemicals of industrial significance. In general, succinic acid is synthesised through chemical methods such as electroreduction or catalytic hydrogenation of maleic acid or paraffin oxidation. However, these chemical routes suffer from various limitations, e.g. poor purity and yield of the organic acid, complexity in their operation and expensive and possible environmental implications. Further, in view of the exponential decay in crude oil reserve and demand for sustainable development, there has been a paradigm shift in succinic acid production through microbial transformation of renewable feedstock. While substantial advancement has been achieved in the area of biotechnological production of succinic acid, much efforts need to be made to achieve

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_15

economic viability. This chapter sheds light on current state of the art of biological production of succinic acid including metabolic pathway, microorganisms, metabolic engineering of potential strains, renewable feedstock, bioprocess development and downstream processing. Based on the existing technological challenges, future research perspective is also discussed.

Keywords

Succinic acid · Metabolic pathway · Genetic modification · Bioprocess development · Scale-up · Downstream processing

Abbreviations

α-KG	α-ketoglutarate
AcCoA	Acetyl-CoA
aceA	Isocitrate lyase
aceB	Malate synthase
Acetyl-P	Acetyl-phosphate
ack	Acetate kinase
ackA	Acetate kinase
acn	Aconitate hydratase
aco	Aconitate hydratase
acs	Acetate-CoA ligase
acsA	*Bacillus subtilis* acetyl-CoA synthase
adh	Alcohol dehydrogenase
ald	Aldehyde dehydrogenase
cat	Acetyl-CoA: CoA transferase
CIT	Citrate
cit	citrate lyase
DHAP	Dihydroxyacetone phosphate
fdh	*Mycobacterium vaccae* Formate dehydrogenase
frd	Fumarate reductase
fruA	Fructose phosphotransferase gene
fum	Fumarase
FUM	Fumarate
fum	Fumarate hydratase
G-3-P	Glyceraldehyde 3-phosphate
gapA	Glyceraldehyde 3-phosphate dehydrogenase
glk	Glucose kinase
gltA	Citrate synthase
GOX	Glyoxylate
icd	Isocitrate dehydrogenase
icl	Isocitrate lyase
ICT	Isocitrate

idh	Isocitrate dehydrogenase
kdh	α-KG dehydrogenase
ldh	Lactate dehydrogenase
maeB	Malic enzyme
MAL	Malate
mdh	Malate dehydrogenase
mgsA	Methylglyoxal synthase
mls	Malate synthase
OAA	Oxaloacetate
oad	Oxaloacetate decarboxylase
pck	PEP carboxykinase
PEP	Phosphoenolpyruvate
pfl	Pyruvate formate lyase
pflB	Pyruvate formate lyase
pfo	Pyruvate-ferredoxin oxidoreductase
poxB	Pyruvate dehydrogenase
pqo	pyruvate:menaquinone oxidoreductase
pta	Phosphate acetyltransferase
pta	phosphotransacetylase
PTS	Phosphotransferase system
pyc	Pyruvate carboxylase
pyk	Pyruvate kinase
pykF	Pyruvate kinase
PYR	Pyruvate
QOH	Menaquinol
sdh	Succinate dehydrogenase
Suc-CoA	Succinyl-CoA
TCA	Tricarboxylic acid

15.1 Introduction

Succinic acid, an intermediate metabolite of the tricarboxylic acid cycle, has emerged as a compound of industrial importance due to its widespread application across a variety of industries. Succinic acid has found uses in the beverage and food industries as an acidity regulator and flavouring ingredient. It is also used as an excipient in the production of pharmaceutical products and in manufacturing of resins, pesticides, dyes, etc. Due to its role as an essential commercial precursor, a lot of research has been directed towards the improvement of the production process of succinic acid. Conventionally, succinic acid has been produced using petroleum-based feedstocks via the catalytic hydrogenation of maleic anhydride. However, the dependence on fossil fuels increases the expense and lowers the sustainability of the process in the long run. Hence, the biological synthesis of succinic acid using

renewable feedstocks has garnered a lot of attention in the recent past. This method is relatively economical as well as sustainable, making it a viable alternative for the existing processes. It involves the fermentation of agricultural substrate raw materials like lignocellulosic hydrolysate, rapeseed meal, corn steep liquor, cane molasses, etc. by various succinic acid-producing bacteria (Guettler et al. 1996; Agarwal et al. 2007; Li et al. 2011). Various techniques have been employed to bring the bio-based production methods at par with the current commercial methods of production. Refinement in downstream processing, substrate optimisation, genetic modification of the microorganisms and scale-up are some of the key research areas where significant focus has been directed. Discussed henceforth is a concise overview of the metabolic pathways and organisms involved in succinic acid production, the bioprocess development parameters, current advancements and market scenario for the production of this compound and a view into the current technological challenges and future perspectives. The ongoing shift towards the production of succinic acid via bio-based methods provides a hope for the adoption of a holistic production process that may take us a step forward from the point of view of economic viability, technological feasibility and overall sustainability.

15.2 Metabolic Pathway of Succinic Acid

Succinic acid is synthesised in the tricarboxylic acid cycle as an intermediate metabolite and also as an end product of anaerobic cultivation of several microorganisms. Major pathways following succinic acid synthesis are (1) reductive and (2) oxidative branch of the tricarboxylic acid cycle and the (3) glyoxylate cycle. Amongst them, the oxidative branch of the tricarboxylic acid cycle mainly produces succinic acid as an intermediate of this pathway, whereas the reductive branch deals with the fermentation process resulting in the production of succinic acid as a mixed acid. In contrast, glyoxylate shunt is followed by certain bacteria (e.g. *Escherichia coli*), fungi (i.e. *Saccharomyces cerevisiae*) and higher plants and also in genetically modified organisms due to its advantage of avoiding the loss of carbons as CO_2 and producing a high concentration of succinic acid under aerobic condition, requiring fewer reducing equivalents.

15.2.1 Anaerobic Fermentation

The reductive branch of the TCA cycle mainly occurs under anaerobic conditions where succinate functions as H-acceptor. In this pathway, a carbon source is converted to phosphoenolpyruvate (PEP) which is then destined to produce succinate following the production of oxaloacetate, malate and fumarate successively requiring 2 mol of NADH per mol of succinate production, shown in Fig. 15.1a.

The key steps involved are:

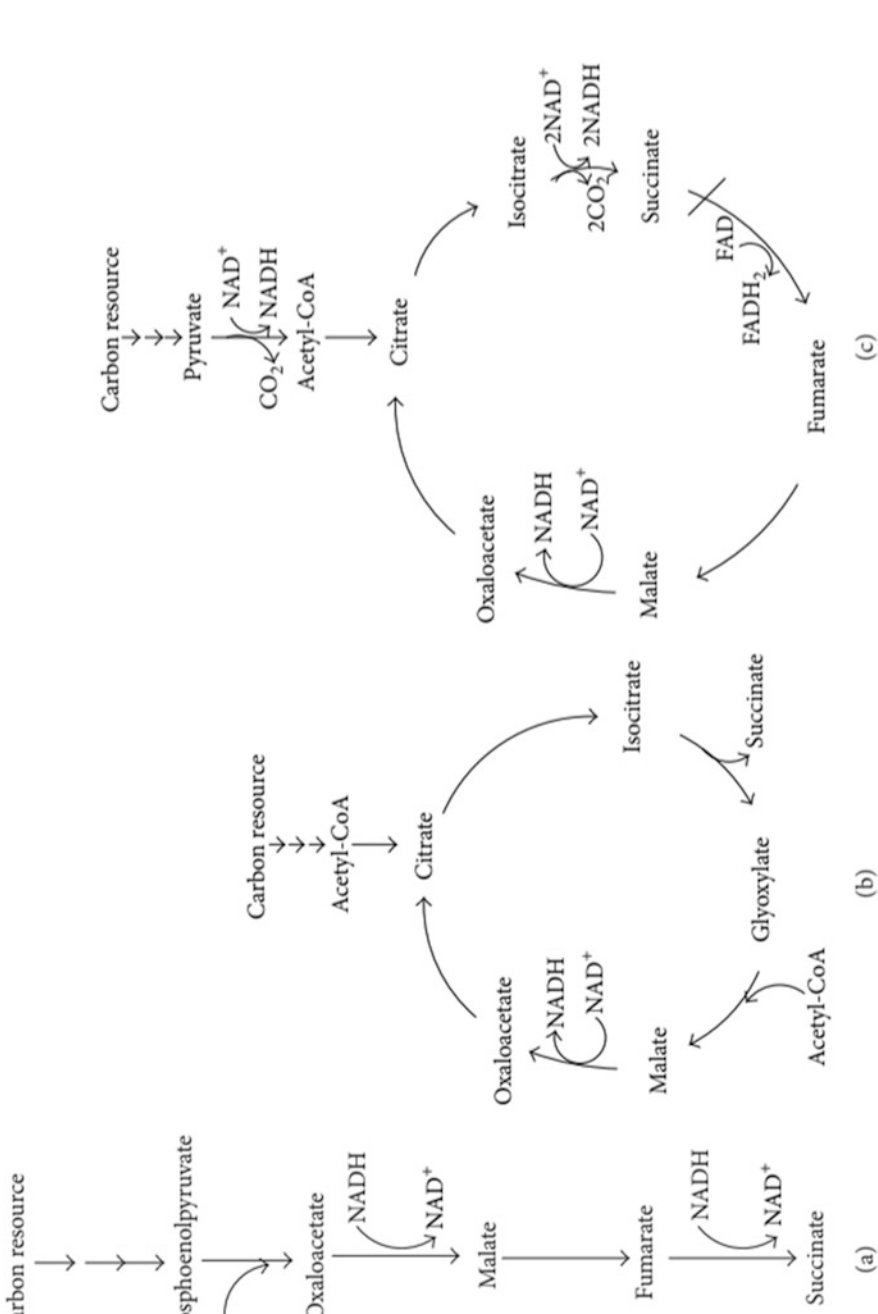

Fig. 15.1 Metabolic pathway of succinate production from (**a**) the reductive branch of the tricarboxylic acid cycle; (**b**) the glyoxylate cycle and (**c**) the oxidative tricarboxylic acid pathway (Cheng et al. 2013)

1. Phosphoenolpyruvate (PEP), an intermediate of the glycolytic pathway, is carboxylated by the enzyme phosphoenolpyruvate carboxylase to form oxaloacetate:

$$\text{Phosphoenolpyruvate} + HCO_3 \rightarrow \text{oxaloacetate} + \text{phosphate}\ [\text{PEP carboxylase}] \quad (15.1)$$

2. NAD-linked malate dehydrogenase catalyses the dehydrogenation reaction to form malate from oxaloacetate:

$$\text{Oxaloacetate} + \text{NADH} + H^+ \rightarrow \text{malate} + NAD^+ [\text{malate dehydrogenase}] \quad (15.2)$$

3. Fumarate is produced from the dehydration of malate catalysed by fumarate hydratase:

$$\text{Malate} \rightarrow \text{fumarate} + H_2O\ [\text{fumarase}] \quad (15.3)$$

4. Finally, fumarate reductase catalyses the reduction of fumarate to produce succinate:

$$\text{Fumarate} + \text{NADH} + H^+ \rightarrow \text{Succinate} + NAD^+ [\text{fumarate reductase}] \quad (15.4)$$

The equation describing the reductive metabolic pathways is as follows:

$$\text{PEP} + CO_2 + 2\,\text{NADH} \rightarrow \text{Succinate} + 2\,NAD^+ + H^+ \quad (15.5)$$

15.2.2 Glyoxylate Shunt

The glyoxylate cycle is an anaplerotic reaction to replenish the molecular pool of the TCA cycle. Due to the lacking of an extra electron benefactor, enacted glyoxylate pathway provides additional NADH to the anaerobic fermentation process and accomplishes a higher yield of succinate. This cycle bypasses the decarboxylation steps of the Krebs cycle by condensing acetyl-CoA with oxaloacetic acid to give citric acid which is subsequently transformed to isocitric acid. This isocitrate is then sliced by isocitritase to produce succinic acid and glyoxylic acid. In many mutant strains, e.g. HL27659k(pKK313) strain of *E. coli*, metabolic modification has been done in a way described later aiming to activate the glyoxylate shunt to enhance succinic acid productivity. This pathway converts 2 mol of acetyl-CoA to 1 mol of succinate as shown in Fig. 15.1b, and the reaction steps are as follows:

1. The condensation reaction of acetyl coenzyme A and oxaloacetate is catalysed by citrate synthase to produce citrate:

$$\text{Oxaloacetate} + \text{Acetyl CoA} + H_2O \rightarrow \text{Citrate} + \text{CoA-SH}\ [\text{citrate synthase}] \quad (15.6)$$

2. Citrate is isomerised into isocitrate by aconitase. Citrate is isomerised through a dehydration step followed by a hydration step:

$$\text{Citrate} \rightarrow \text{Isocitrate}\ [\text{aconitase}] \quad (15.7)$$

3. The disunion of isocitrate into glyoxylate and succinate is catalysed by isocitrate lyase:

$$\text{Isocitrate} \rightarrow \text{Glyoxylate} + \text{Succinate}\ [\text{isocitrate lyase}] \quad (15.8)$$

The equation describing the glyoxylate pathway is as follows:

$$2\text{Acetyl CoA} + 2H_2O + NAD^+ \rightarrow \text{Succinate} + 2\text{CoASH} + \text{NADH} + 2H^+ \quad (15.9)$$

15.2.3 Aerobic Pathway

Under aerobic conditions, which describe this oxidative TCA cycle, acetyl-CoA is converted to isocitrate, citrate and succinate which is in turn transformed into fumarate by succinate dehydrogenase as depicted by a schematic representation in Fig. 15.1c. Here deactivation of sdhA gene is necessary to prevent the transformation of succinate to fumarate in order to produce succinic acid. The key steps involved in this pathway are mentioned below:

1. Pyruvate dehydrogenase complex catalyses non-reversible oxidative decarboxylation, to produce CO_2 and acetyl-CoA:

$$\text{Pyruvate} + \text{CoA-SH} + NAD^+ \rightarrow \text{Acetyl CoA} + CO_2 + NADH^+\ [\text{pyruvate dehydrogenase}] \quad (15.10)$$

2. Condensation of acetyl-CoA and oxaloacetate into citrate is catalysed by citrate synthase:

$$\text{Oxaloacetate} + \text{Acetyl CoA} + H_2O \rightarrow \text{Citrate} + \text{CoA-SH [citrate synthase]} \quad (15.11)$$

3. The reversible conversion of citrate to isocitrate is catalysed by aconitase:

$$\text{Citrate} \rightarrow \text{Isocitrate [aconitase]} \quad (15.12)$$

4. Isocitrate dehydrogenase then catalyses oxidative decarboxylation of isocitrate to form α-ketoglutarate:

$$\text{Isocitrate} + NAD^+ \rightarrow \alpha\text{-ketoglutarate} + NADH^+ + CO_2 \text{ [isocitrate dehydrogenase]} \quad (15.13)$$

5. α-Ketoglutarate is transformed to CO_2 and succinyl-CoA by α-ketoglutarate dehydrogenase complex; NAD acts as electron acceptor and CoA as the carrier of the succinyl group:

$$\alpha\text{-ketoglutarate} + \text{CoA-SH} + NAD^+ \rightarrow \text{Succinyl CoA} + CO_2 \text{ [α-ketoglutarate dehydrogenase]} \quad (15.14)$$

6. Succinic thiokinase or succinyl-CoA synthetase produces succinate from succinyl-CoA:

$$\text{Succinyl-CoA} + GDP + Pi \rightarrow \text{Succinate} + \text{CoA-SH} + GTP \text{ [succinyl-CoA synthetase]} \quad (15.15)$$

Equation of this oxidative pathway is given below:

$$2\text{Pyruvate} + 2H_2O + 3NAD^+ \rightarrow \text{Succinate} + 3NADH^+ + 2CO_2 \quad (15.16)$$

The key enzymes responsible for the synthesis of succinic acid are malate dehydrogenase (mdh), fumarase (fum), succinate dehydrogenase (sdh), phosphoenolpyruvate carboxylase (PEPC), pyruvate carboxylase (pyc), pyruvate dehydrogenase (pdh) and phosphoenolpyruvate carboxykinase (PEPCK).

15.3 Wild-Type Microorganisms for the Production of Succinic Acid

Succinic acid being a transitional metabolite of the tricarboxylic acid cycle is produced ubiquitously by many organisms including microbes, plants and animal cells. However, for industrial production fermentative production by microbes has been preferred, and mainly three metabolic routes are operational, i.e. reductive,

oxidative and glyoxylate pathway. Under anaerobic conditions, the reductive TCA (rTCA) cycle is majorly operational with fumarate reductase, fumarase, malate dehydrogenase and PEP carboxykinase being critical flux regulators. Further, NADH also influences succinic acid production as maximal theoretical yield of 2 mol of succinic acid per mol of glucose can only be achieved with fixing of 1 mol of succinic acid with 1 mol of carbon dioxide and 2 mol of NADH (Dai et al. 2020). In the TCA cycle, operative under aerobic conditions, the theoretical yield obtained is 1 mol of succinic acid per mol of glucose. The carbon loss in oxidative decarboxylation reaction confines the yield of succinic acid to 1.71 mol per mol glucose in the glyoxylate pathway. Since these metabolic pathways are regulated by series of genes, various approaches to engineer the metabolic machinery of microbes have been pursued to upsurge succinic acid yield. Further, the accumulation of by-products leads to decreased yields and high purification costs in downstream processing. Thus, recombinant DNA technology has been used for deletion, insertion or overexpression of specific genes operational in the particular metabolic pathway of targeted strain. Therefore, *Escherichia coli*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica* have been successfully subjected to genetic manipulations for succinic acid production in high titres (Li et al. 2018, 2019a, b; Ferone et al. 2019; Dai et al. 2020).

Nonetheless, wild-type microbes have also been characterised extensively for the production of succinic acid. Though bacterial strains remain the prominent choice for bio-based production, eukaryotic systems in both natural and engineered forms have been employed for bio-based production. Amongst wild-type eukaryotic producers, the most notable fungi species are *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium simplicissimum*, *Penicillium viniferum*, *Fusarium* sp., *Byssochlamys nivea*, *Paecilomyces variotii* and *Lentinus degener* (Mancini et al. 2020; Lu et al. 2021). The culture consortium of *A. niger*, *Trichoderma reesei* and *Phanerochaete chrysosporium* was reported to produce 61.12 g/l succinic acids by utilising a two-phase slurry fermentation approach (Alcantara et al. 2017).

The most commonly reported strains amongst bacteria are *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Basfia succiniciproducens*, *Corynebacterium crenatum* and *Mannheimia succiniciproducens* (Jiang et al. 2017; Mancini et al. 2020). These microbes have been reported to ferment a variety of substrates, viz. pure sugars (glucose, xylose, arabinose, mannose) as well as hydrolysate of biomass feedstocks (corncob, rice husks, oil palm frond bagasse, sugarcane bagasse, etc.). Amongst the above microbes, the facultative anaerobic, non-pathogenic and Gram-negative *A. succinogenes* has been reported as the most potent producer due to no requirement of aeration and ability to utilise C5, C6 carbohydrates as well as other carbon substrates, viz. glycerol, molasses, starch, etc., and produce high yield of succinic acid. It is also reported to exhibit high tolerance to toxicants like HMF and furfural in hydrolysates, form biofilms, carry out effective consumption and fixation of CO_2 and exhibit effective performance in scalable biorefinery systems (Dessie et al. 2018a, b; Zhang et al. 2020). In a first report, nitrogen-rich palm trunk sap could be used as the sole substrate by *A. succinogenes* with succinic acid production of 0.54 g/g, which could effectively reduce the cost of

fermentative media's expensive constituents (Bukhari et al. 2019). Similarly, *A. succiniciproducens* have also been reported to utilise a broad range of feedstocks though with different yields, viz. 32.2 g/l and 15.3 g/l of succinic acid were synthesised by *A. succiniciproducens* by utilising glucose and galactose, respectively (Dai et al. 2020). The utilisation of arabitol, mannitol, lactose, maltose, xylose, fructose and glycerol for synthesis of succinic acid has also been carried out by Gram-negative and facultative anaerobic *M. succiniciproducens* (Dai et al. 2020; Mancini et al. 2020).

Since the utilisation of pure sugars escalates the production cost, the approach of valorising starchy as well as lignocellulosic waste feedstocks is garnering attention though its upscale has more limitations than simple substrates. Further, the hydrolysate of renewable feedstocks contains complex energy sources, and therefore, the assimilation of these sources by new microbial strains for succinic acid production is highly desirable. Recently, a new bacterial strain *Enterobacter aerogenes* LU2 identified from cow rumen was reported to produce 57.7 g/l succinic acids from whey permeate (Szczerba et al. 2020a). Its complete genomic analysis along with functional studies confirmed the production of succinic acid by reductive TCA as well as glyoxylate pathway along with several other desirable traits for its potential as the metabolic factory for biological production of bulk chemicals (Szczerba et al. 2020b). In another study, the production of 43.7 g/l succinic acid was achieved by *A. succinogenes* by utilising waste bread as substrate (Leung et al. 2012). Similarly, the valorisation of cake and pastry waste by *A. succinogenes* yielded succinic acid titre in the range of 24.8–31.7 g/l (Zhang et al. 2013). Another starch-rich biomass duckweed (*Landoltia punctata*) was utilised as feedstock by *Actinobacillus succinogenes*, resulting in the production of 57.85 g/l of succinic acid (Shen et al. 2016). *Corynebacterium crenatum* could produce 43.6 g/l of succinic acid from hydrolysate of wheat bran (Chen et al. 2013).

The utilisation of a lignocellulosic substrate, i.e. ionic liquid-pretreated pine wood, led to the production of 20.7 g/l succinic acids by *Actinobacillus succinogenes* 130Z (Wang et al. 2014). Similarly, other lignocellulosic wastes such as wheat straw (Zheng et al. 2009), corn fibre (Chen et al. 2010), corn stover (Zheng et al. 2010), rapeseed meal (Chen et al. 2011), corn stalk and cotton stalks (Li et al. 2010b) have been effectively used for the production of succinic acid by *Actinobacillus succinogenes*. The hydrolysate of energy crop *Arundo donax* could be fermented by *Basfia succiniciproducens* at a pilot scale with a yield of 0.75 mol/mol (Cimini et al. 2016). In another study, *B. succiniciproducens* produced succinic acid with 46% yield by using kitchen waste hydrolysate as feedstock (Babaei et al. 2019). The immobilised cells of *Corynebacterium glutamicum* were able to produce 22.5 g/l succinic acids by using cassava bagasse hydrolysate (Shi et al. 2014). Overall, for the effective valorisation of lignocellulosic into succinic acid, microbial co-cultivation and consolidated bioprocessing (CBP) are being extensively investigated to overcome the challenges of the recalcitrance of biomass (Lu et al. 2021).

Summarily, though natural strains for succinic acid production are being probed to avoid the complexity of gene editing, their limitations due to auxotrophic nature,

sensitivity to pH and other inhibitors along with diverse by-product formation divert interest towards metabolic flux channelisation by genetic engineering tools to create sustainable biorefining strategies for succinic acid production.

15.4 Genetically Modified Microbes for the Production of Succinic Acid

Capnophilic rumen bacteria like *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes* and *Mannheimia succiniciproducens* are the high-yielding natural producers of succinic acid due to their efficiency in fixing CO_2, and they have the highest enzymatic levels of malate dehydrogenase, PEP carboxykinase and fumarase relative to other organisms, i.e. *Escherichia coli*, *Basfia succiniciproducens*, S*accharomyces cerevisiae* and *Corynebacterium glutamicum*. These natural succinic acid-producing bacteria have been exclusively studied through metabolic engineering, optimisation of fermentation technologies and separation technology. Genetic modification has been done mainly focusing on:

1. *The inhibition of end product other than succinic acid:* It is necessary to block the enzymes in the competing metabolic processes where succinic acid is synthesised as a mixed acid with other lactic, formic and acetic acid mainly under anaerobic fermentation to enhance succinic acid productivity.
   Example: NZN11 strain of *E. coli* was genetically modified with the removal of lactate dehydrogenase (*ldhA*) and pyruvate formate lyase (*pfl*) genes responsible for lactic and formic acid production, respectively, to prevent these acids formation by *E. coli* under anaerobic conditions (Wang et al. 2009b).
2. *Changing the redox equivalent:* The number of additional reducing equivalents is directly proportional to the succinic acid yield. To address this, BOL3/pan6-gap strain of *C. glutamicum* was metabolically engineered with the chromosomal incorporation of NAD^+-linked formate dehydrogenase from *M. vaccae* and the overexpression of glyceraldehyde 3-phosphate dehydrogenase to elevate the ratio of NADH to NAD^+ (Ito et al. 2014). Another example described later in this section is the development of SBS550MG (pHL413KF1) strain to increase the NADH availability.
3. *The different substrates utilising C-source:* The most important fact needed to consider before choosing a carbon source is that the glycerol is an abundant C-source and inexpensive and has the ability to produce twofold more reducing equivalents (i.e. NADH, $FADH_2$ and NADPH) relative to other sugars like glucose (Yazdani and Gonzalez 2007). But the limitation of using glycerol is the growth rate under anaerobic conditions. The solution to this limitation has been acquired by the simultaneous use of sucrose due to its higher utilisation and cell growth rate. Examples are the development of PALFK and PALKG strains, explained later.

Many organisms have been engineered for succinic acid production including the model organism *E. coli*. A brief description of the enzyme genes engineered has been discussed below.

15.4.1 *Actinobacillus succinogenes*

Actinobacillus succinogenes 130Z can use a broad array of carbon substrates including glycerol, arabinose, lactose, fructose, sucrose and glucose for producing succinic acid as a product of mixed acid with acetic and formic acid through anaerobic fermentation (Guettler et al. 1996). For overcoming this, FZ53 strain, a mutant of 130Z, was selected having the capacity to produce 105.8 g/l succinic acids with the yield of 1.22 mol/mol glucose (Guettler et al. 1996). Physiological and genetic studies have distinguished five key catalysts, in particular, malate dehydrogenase (*mdh*), PEP carboxykinase (*pck*), malic compound (*sfc*), fumarate reductase (*frd*) and fumarase (*fum*) that account for the higher yield of succinic acid production in *Actinobacillus succinogenes*.

15.4.2 *Anaerobiospirillum succiniciproducens*

Anaerobiospirillum succiniciproducens produces mixed acid of acetic acid and succinic acid as a major product with lactic acid and ethanol as a by-product under anaerobic fermentation (Bretz 2015). It has the ability to consume a broad range of carbon substrates along with glycerol, which is transported by facilitated diffusion and ultimately leads to the conversion of phosphoenolpyruvate to succinic acid. Here, utilisation of glycerol is more preferable due to two reasons: (1) it provides more redox equivalent and (2) it reduces acetic acid accumulation. This process results in 6.5 times greater production of succinic acid than obtained from glucose. The key enzymes involved are phosphoenolpyruvate carboxykinase (PEPCK), a carbon dioxide-fixing enzyme, catalysing ATP-based reversible decarboxylation of oxaloacetate to produce PEP, oxaloacetate decarboxylase and pyruvate kinase (Lee et al. 2001).

15.4.3 *Corynebacterium glutamicum*

The metabolic pathway followed by wild-type *Corynebacterium glutamicum* involves the production of mixtures of lactic acid, acetic acid and succinic acid only when cell growth is stopped under anaerobic conditions utilising glucose as a major substrate. Therefore, it is necessary to downregulate certain pathways to bypass the lactic and acetic acid formation and obtaining a higher concentration of succinic acid. Metabolically engineered *C. glutamicum* ΔldhpCRA717 strain produced 146 g/l/h of succinic acid with a yield of 1.4 mol/mol glucose. In this mutant strain, pyruvate decarboxylase gene was overexpressed, and the lactate

dehydrogenase gene was inhibited to enhance pyruvate to oxaloacetic acid conversion (Okino et al. 2008). Further, acetate kinase (*ackA*), phosphotransacetylase (*pta*), acetyl-CoA/CoA-transferase (*cat*), pyruvate/menaquinone oxidoreductase (*pqo*) and lactate dehydrogenase (*ldhA*) genes were removed to decrease acetic acid and lactic acid accumulation along with the chromosomal integration of pyruvate carboxylase. This engineered strain, BOL3/pan6-gap, contained chromosomally integrated NAD^+-linked formate dehydrogenase from *M. vaccae* as mentioned earlier (Litsanov et al. 2012). Finally, in this strain glyceraldehyde 3-phosphate dehydrogenase was overexpressed only to increase glucose uptake under anaerobic conditions and to uplift the NADH/NAD^+ ratio. This resulted in 134 g/l of succinic acid with a yield of 1.67 mol/mol glucose, and both the strains could be aerobically precultured (Jojima et al. 2010; Litsanov et al. 2012). The aerobic process is more preferable due to its faster carbon flux, higher growth rate and higher product formation. Considering these factors, another ZX1(pEacsAgltA) strain was engineered with the removal of succinate dehydrogenase complex (*sdhCAB*, *ldhA*, *pqo*, *cat* and *pta*) genes to produce succinic acid aerobically (Zhu et al. 2013). This strain was developed by overexpressing acetyl-CoA synthase coded by the *acs* gene from *Bacillus subtilis* for efficient succinic acid production without accumulating acetic acid. Finally, the promoter of *pyc* and *ppc* was replaced with the stronger promoter (P_{SOD}) (Zhu et al. 2013). This engineered *C. glutamicum* reported the highest performance values with a titre of 28.5 g/l succinic acids with a yield of 0.63 mol/mol glucose (Kuhnert et al. 2010).

15.4.4 *Escherichia coli*

E. coli, in contrast, can produce succinic acid under both aerobic and anaerobic conditions but in a very small amount utilising glucose as a C-source. Under aerobic conditions wild-type *E. coli* forms succinic acid only as an intermediate of the TCA cycle by inactivating the succinate dehydrogenase encoding gene, *sdhA*, whereas in the reductive branch of the tricarboxylic acid cycle, it synthesises succinic acid as a minor product of mixed acids. Overexpression of PEPC gene increased succinic acid production 3.5-fold in *E. coli* (Millard et al. 1996). In an engineered *E. coli* strain, PEPC or pyruvate carboxylase (PYC) and native pantothenate kinase (PANK) genes were overexpressed and resulted in the increased carbon flux towards succinic acid production from phosphoenolpyruvate and pyruvate. Overexpression of carbonic anhydrase coding gene, the *eca* gene from cyanobacterium *Anabaena* sp. 7120 in *E. coli*, led to an increase of PEPC activity 2.1 times with the yield from 0.19 to 0.39 mol/mol glucose (Wang et al. 2009a). Under anaerobic conditions, it is necessary to prevent formic and lactic acid formation in order to uplift succinic acid production. Therefore, NZN111/pTrc99A-mdh strain of *E. coli* was genetically modified with the deletion of *ldhA* and *pfl* genes encoding lactate dehydrogenase and pyruvate formate lyase and overexpression of native malate dehydrogenase leading to the production of 31.9 g/l of succinic acid with a yield of 1.19 mol/mol glucose (Wang et al. 2009b). Another AFP111 strain was designed with the spontaneous

chromosomal mutation of glucose phosphotransferase coding *ptsG* gene along with the removal of lactate dehydrogenase and pyruvate formate lyase encoding *ldhA* and *pfl* genes, respectively, and the succinic acid production was increased by the yield of 1 mol/mol glucose (Vemuri et al. 2002). Furthermore, since glyoxylate shunt is more preferable, another strain was engineered with the deletion of transcriptional repressor gene *iclR* and *pta-ack*, *ldhA* and *adhE* genes from the central metabolic pathway. This SBS550MG strain has the ability to produce 40 g/l of succinic acid with a yield of 1.6 mol/mol glucose (Sánchez et al. 2005). Moreover, two strains were developed, i.e. SBS550MG (pHL413KF1) and SBS550MG (pHL413Km), with the introduction of pyruvate carboxylase (*pycA*) from *L. lactis* and NAD +- -linked formate dehydrogenase (*fdh1*) from *C. boidinii*, respectively. SBS550MG (pHL413KF1) shows 6% increased succinic acid production compared to SBS550MG (pHL413KF1) strain (Balzer et al. 2013). Genes of the glucose transferase system (*ptsG*), TCA cycle (*sdhAB*, *iclR*) and competing by-product-forming pathways (*poxB*, *pta-ackA*) were deleted with the overexpression of *ppc* gene from *S. vulgare* in the HL27659k (pKK313) strain. This mutant strain of *E. coli* uses glyoxylate cycle to synthesise 58.3 g/l of succinic acid with the yield of 1.08 g/l/h under fully aerobic condition (Lin et al. 2005a, b). KJ134 strain was genetically modified by disrupting *pta* gene to reduce acetic and pyruvic acid in order to synthesise 71.6 g/l of succinic acid with the yield of 1.53 mol/mol glucose (Jantama et al. 2008). Another strain ZJG13/pT184pyc was constructed on the basis of CASOP (computational approach for strain optimisation), in silico analysis by deleting *pta-ackA*, *poxB*, *iclR*, *sdhA* and *mgsA* genes and overexpressing *pyc* gene resulting in the succinic acid productivity of 2.75 mmol/g dry cell per hour (Yang et al. 2014).

15.4.5 *Mannheimia succiniciproducens*

In comparison to the previous organisms, *Mannheimia succiniciproducens* MBEL55E utilise glycerol less efficiently than other carbon sources such as glucose, xylose, sucrose, mannitol, arabitol, maltose, fructose and lactose (Lee et al. 2002). It synthesises succinic acid as the primary fermentative end product, while acetic, lactic and formic acid as a by-product (Song and Lee 2006). So, pyruvate dissimilating enzymes were needed to disrupt or remove the by-product generation. Using medium defined chemically containing six indispensable necessary components with four vitamins (pyridoxine, pantothenate, nicotinic acid and biotin) and amino acids methionine and cysteine, succinic acid concentration, yield and productivity increased by a significant proportion compared to complex media, and the lactic, acetic and formic acid production was reduced to 30% (Song et al. 2008). In wild type *Mannheimia succiniciproducens* MBEL55E *pckA* encoding PEPCK enzyme played an important role in C_3 to C_4 conversion, and hence succinic acid production was 10.5 g/l under anaerobic batch fermentation (Lee et al. 2006). Genetically modified *M. succiniciproducens* PALK strain was developed on the basis of in silico network simulation. In this mutant strain, *ldhA*, *pta* and *ackA* genes

were deleted, resulting in the titre of 45.8 g/l of succinic acid with the yield of 1.32 mol/mol of glucose (Lee et al. 2009). This strain had the ability to produce fewer by-products in comparison to LPK7 in which lactate dehydrogenase, pyruvate formate lyase, phosphate acetyltransferase and acetate kinase were removed (Lee et al. 2009). Fructose phosphotransferase (*fruA*) gene was obliterated from PALK strain to constitute PALFK, having productivity of 68.4 g/l of succinic acid with a yield of 1.57 mol/mol (Lee et al. 2014). Another strain of PALK, PALKG, with overexpression of glycerol kinase (*glpK*) from *E. coli* can produce 64.7 g/l of succinic acid with a yield of 1.39 mol/mol. As stated previously these two strains use substrate co-utilisation technique to produce a higher amount of succinic acid without by-product formation using glycerol and sucrose as C-source.

Succinity, a joint venture between Corbion Purac and BASF, has a yearly potential of 10,000 metric tons and is synthesising commercial quantities of bio-succinic acid for the international market. The plant is registered in 2013 and is located in Montmeló, Spain. It uses *Basfia succiniciproducens*, identified from the rumen of German cow which shares 95% protein homology and identical G-C content (42.5%) with *M. succiniciproducens* MBEL55E (Kuhnert et al. 2010). A mutant strain of this bacterium (DD1) with double deletion of *ldhA* and *pflD* synthesised succinic acid at a yield of 1.08 mol/mol glucose.

15.4.6 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, an alternative host, could produce succinic acid up to 2 g/l only as a by-product of ethanol fermentation considering glucose as C-source (Whiting 1976). Therefore, a mutant strain was metabolically engineered by knocking out fumarate reductase (*osm1*) gene leading to the production of 1.5-fold higher titre of succinic acid (Whiting 1976). Disruption of succinate dehydrogenase (*sdh1* and *sdh2*) and alcohol dehydrogenase genes (*adh1*, *adh2*, *adh3*, *adh4* and *adh5*) showed 20 times greater succinic acid production with the yield of 0.217 mol/mol glucose through the TCA cycle under aerobic conditions (Ito et al. 2014). Metabolically modified *Saccharomyces cerevisiae* strain with the deletion of isocitrate dehydrogenase genes (*idh1* and *idp1*) and the *sdh1* and *sdh2* genes could set the metabolic flux through glyoxylate pathway to produce 3.62 g/l of succinic acid with a yield of 0.11 mol/mol glucose (Raab et al. 2010). In another mutant of *S. cerevisiae* strain, glycerol-3-phosphate dehydrogenase gene was obliterated, and its intrinsic fumarate hydratase gene (*fum1*) was exchanged with *E. coli* fumarate hydratase gene (*fumC*) along with the addition of *pyc2*, *mdh3* and *frd1* genes coding pyruvate carboxykinase 2, malate dehydrogenase and fumarate reductase, respectively. This strain had productivity of 13 g/l of succinic acid with a yield of 0.21 mol/mol glucose (Yan et al. 2014).

Thus, the biological production process for succinic acid employed microbes that used the reductive branch of the tricarboxylic acid cycle; carbon dioxide is fused to produce succinic acid. Microbes such as Gram-positive bacteria, yeast and fungi have been designated and studied for the production of succinic acid. Table 15.1

Table 15.1 Succinic acid production from genetically modified/mutated organisms

SA-producing strain	Carbon source	Fermentation type	Titre (g/l)	References
C. glutamicum BOL3/pAN6-gap; Δ*cat*, Δ*ldhA*, Δ*pqo*, Δ*pta-ackA*, +*pyc*, +*fdh*, +*gapA*	Glucose	Fed-batch	134	Litsanov et al. (2012)
C. glutamicum R mutant/pCRA717; ΔldhA, +pyc	Glucose	Fed-batch	146	Okino et al. (2008)
E. coli AFP111/pTrc99a-pyc; Δ*pflAB*, Δ*ptsG*, Δ*ldhA*, +*pyc*	Glucose	Dual-phase fed-batch	99.2	Vemuri et al. (2002)
E. coli SBS550MG; Δ*adhE*, Δ*ldhA*, Δ*iclR*, Δ*pta-ackA*, +*pyc*	Glucose	Repeated fed-batch	40	Sánchez et al. (2005)
M. succiniciproducens MBEL55E; wild type	Glucose	Batch	10.51	Song et al. (2007)
M. succiniciproducens PALK; ΔldhA, Δpta-ackA	Glucose, glycerol	Fed-batch	45.79	Lee et al. (2009)
S. cerevisiae PMCFfg; Δ*fum1*, Δ*gpd1*, Δ*pdc1*, Δ*pdc5*, Δ*pdc6*, +*fumC*, +*pyc2*, +*mdh3*, +*frd1*	Glucose	Dual-phase batch	13	Yan et al. (2014)
S. cerevisiae AH22ura3; Δ*sdh1*, Δ*sdh2*, Δ*idh1*, Δ*idp1*	Glucose	Shake-flask culture	3.62	Agren et al. (2013)
I. orientalis SD108 *Strain Io−ura3−+−SA*	Glucose	Batch	11.63	Xiao et al. (2014)
I. orientalis SD108 Io−ura3−+−ura3	Glucose	Batch	1.43	Xiao et al. (2014)

summarises the production of succinic acid by different genetically modified/mutated strains.

15.5 Bioprocess Development

15.5.1 Substrate

Substrates play a vital role in the growth and product-synthesis metabolism of microorganisms. Lately, researchers are looking for low-cost, renewable and sustainable substrates as an alternative for conventional chemical sources of carbon, nitrogen and other minerals. Sugar industries are known to produce cane molasses as a by-product, which is a rich source of sugars, nitrogenous compounds, vitamins, etc. Liu et al. (2008) outlined the synthesis of 55.2 g/l of succinic acid from *Actinobacillus succinogenes* using cane molasses as substrate in fed-batch operation. In general, microorganisms have the potential to utilise and convert the nutrients

(starch and proteins) present in food wastes into value-added products. *A. succinogenes* has been reported to utilise hydrolysate from waste bread as the substrate for succinic acid production. This study produced 47.3 g/l of succinic acid, equivalent to a yield of 0.55 g succinic acid/g bread (Leung et al. 2012). Authors have also reported the utilisation of starchy crops, such as cassava roots (25–30 w/w % of starch), as feedstock for the synthesis of succinic acid. Thuy et al. (2017) reported the synthesis of 151.44 g/l of succinic acid using enzymatically saccharified cassava roots as substrate under fed-batch operation.

Lignocellulosic biomass is a promising low-cost substrate owing to its high sugar content. These sugars can be easily extracted for the fermentation process by selecting effective pretreatment and hydrolysis methods. Production of succinic acid has been reported from *Miscanthus* × *giganteus*, a perennial, non-food biomass crop. Efficient pretreatment and enzymatic hydrolysis of *Miscanthus* biomass could release up to 93.1% glucose and 69.2% xylose, in the hydrolysate, which was fermented by *Actinobacillus succinogenes* resulting in 75–82% yield of succinic acid (Dąbkowska et al. 2019). Luthfi et al. (2019) reported an advanced coconut shell activated carbon-based homogeneous solid dispersion approach for immobilisation of lignocellulosic biomass (oil palm fronds) and continuous synthesis of succinic acid thereof using *A. succinogenes*.

Agricultural wastes such as sugarcane bagasse are a rich source of cellulose and hemicellulose that can be hydrolysed into a significant quantity of fermentable carbon substrates. Ong et al. (2019) reported the synthesis of 33.2 g/l of succinic acid by *Yarrowia lipolytica* through co-utilisation of xylose and glucose from hydrolysed sugarcane bagasse. Another low-cost and renewable source of fermentable sugars is the waste from the textile industries. Textile wastes are known to contain up to 35–40% of cellulose fibre and hence act as a suitable feedstock for the synthesis of biofuels and organic acids. Pretreated and hydrolysed textile waste requires another stage of treatment to remove the colours/dye from the waste, before utilising the hydrolysate for fermentation. Li et al. (2019a, b) have outlined an effective approach for the elimination of colours/dyes from waste-textile hydrolysate using 2 w/w% biochar. The resulting decolourised hydrolysate was used for fermentation by *Yarrowia lipolytica* in an in situ fibrous bed bioreactor leading to the synthesis of 28.8 g/l of succinic acid.

Fruit and vegetable wastes are an excellent source of carbohydrates, fats and proteins. Dessie et al. (2018a, b) reported an approach to utilise fruit and vegetable wastes as a substrate for succinic acid production. In this study, fungal strains (*A. niger* and *R. oryzae*) were used to produce an enzyme cocktail through solid-state fermentation of the fruit and vegetable wastes. The fungal enzyme cocktail was further utilised for hydrolysing the fruit and vegetable wastes; by fermenting the resultant hydrolysate *A. succinogenes* was able to produce 27.03 g/l of succinic acid. Authors have also reported succinic acid production using macroalgae feedstock. Macroalgae as a substrate are better than lignocellulosic biomass and other equivalent alternatives since it requires milder pretreatment methods for extracting fermentable sugars. This is attributed to the absence of hemicellulose and lignin components in the algal cell which facilitates easy depolymerisation of the biomass.

Fermentation of macroalgae (*Laminaria japonica*) hydrolysate (glucose and mannitol) by engineered *E. coli* led to the production of 17.44 ± 0.54 g/l of succinic acid (Bai et al. 2015). Olajuyin et al. (2016) reported the production of 22.40 ± 0.12 g/l succinic acid through the fermentation of *Palmaria palmata* (red macroalgae) hydrolysate (galactose and glucose) by engineered *E. coli*. Coconut water (*Cocos nucifera*) is known to contain a significant quantity of fermentable sugars, such as sucrose, fructose and glucose. Olajuyin et al. (2019) reported the production of 11.78 ± 0.02 g/l of succinic acid from engineered *E. coli* using coconut water as substrate.

The substrate for a bioprocess is one of the vital factors which determine the yield as well as the cost of the process. Therefore, the selection of suitable substrates is crucial for making a bioprocess sustainable and economical. Table 15.2 summarises the production of succinic acid from different wild-type strains through utilisation of various substrates.

15.5.2 Physicochemical Parameters

In a typical bioprocess, the growth of biomass and formation of the products are significantly influenced by physicochemical factors such as temperature, pH, inoculum density, substrate concentrations, etc. Therefore, process development directed towards maximal production of biomass and the targeted product should encompass process design and optimisation of the fermentation media and other influential conditions. A combinatorial influence of optimised parameters would facilitate the yield of output with maximum response.

Isar et al. (2006) reported the effect of optimising several physicochemical parameters such as carbon source, nitrogen source, pH, inoculum size, temperature, agitation rate, etc. on the synthesis of succinic acid by *Bacteroides fragilis*. The optimum values of these parameters were found to be 1.5% glucose (carbon source), 2.5% tryptone (nitrogen source), 1.5% sodium carbonate, agitation rate of 100 rpm, 37 °C temperature, pH 7.0 and an inoculum density of 4%. When the fermentation was carried out under these optimum conditions, the production titre of succinic acid enhanced from 0.70 g/l (in 60 h) to 5.4 g/l (in 48 h). Under these optimised conditions, when the fermentation was scaled up in a 10-l bioreactor, the succinic acid titre of 12.5 g/l was achieved, which was 12 times higher than the titre obtained under un-optimised conditions. This study also highlights the effect of sodium carbonate as a pH regulator and an enzyme co-factor (due to Na^+ ions) and as a source of carbon dioxide (due to CO_3^{2-} ions). Another study reported the optimisation of the concentrations of magnesium carbonate, yeast extract and cane molasses, for the synthesis of succinic acid by *A. succinogenes.* Under optimised concentrations, a maximum succinic acid titre of 57.43 ± 0.86 g/l was obtained, which was further scaled up to 64.34 g/l in a 1.3-l stirred tank bioreactor under fed-batch operation (Shen et al. 2014). Akhtar et al. (2020) reported RSM-based optimisation of cellulase loading, pH and temperature, for the succinic acid production by *A. succinogenes* from oil palm empty fruit bunches utilising simultaneous

Table 15.2 Succinic acid production by different wild-type strains

Strain	Substrate	Fermentation type (reactor used, if any)	Titre/yield/ productivity	Reference
A. succinogenes 130Z	Corn steep liquor, glucose	Batch	79 g/l	Guettler et al. (1996)
A. succinogenes FZ53	Corn steep liquor, glucose	Batch	105.8 g/l	Guettler et al. (1996)
A. succiniciproducens ATCC29305	Corn steep liquor, glucose	Batch	18 g/l	Lee et al. (2000)
A. succiniciproducens ATCC53488	Glucose	Continuous (membrane bioreactor electrodialysis)	83 g/l	Meynial-Salles et al. (2008)
C. glutamicum R	Glucose	Fed-batch	23 g/l	Okino et al. (2005)
Y. lipolytica PSA02004	Glucose, xylose	Batch	28.2 ± 0.6 g/l	Ong et al. (2019)
E. coli Suc460	Glucose, methanol	Batch	68.54 ± 1.83 g/l	Zhang et al. (2018)
E. coli Suc460	Glucose, methanol	Fed-batch	68.75 ± 1.74 g/l	Zhang et al. (2018)
E. coli Suc460	Glucose	Batch	60.82 ± 1.52 g/l	Zhang et al. (2018)
A. succinogenes 130Z	Glycerol	Batch	2.13 g/l/h	Carvalho et al. (2014)
A. succinogenes 130Z	Glycerol	Fed-batch	2.31 g/l/h	Carvalho et al. (2014)
A. succinogenes	Rapeseed meal (RSM)	Batch	15.5 g/l	Chen et al. (2011)
A. succinogenes NJ113	Lignocellulosic hydrolysate	Batch	56.4 g/l	Li et al. (2011)
A. succinogenes NJ113	Cane molasses	Batch	50.6 g/l	Li et al. (2011)
A. succinogenes	Corn fibre	Stirred bioreactor	0.99 g/l/h	Zheng et al. (2010)
E. coli	Softwood hydrolysates	Batch	42.2 g/l	Hodge et al. (2009)

(continued)

Table 15.2 (continued)

Strain	Substrate	Fermentation type (reactor used, if any)	Titre/yield/ productivity	Reference
E. coli	Cane molasses	Batch	26 g/l	Agarwal et al. (2007)
M. succiniciproducens MBEL55E	Xylose and glucose	Continuous	3.19 g/l/h	Kim et al. (2004)
M. succiniciproducens MBEL55E	Xylose and glucose	Batch	1.17 g/l/h	Kim et al. (2004)
M. succiniciproducens MBEL55E	Whey, corn steep liquor	Batch	1.18 g/l/h	Lee et al. (2003)

saccharification and fermentation approach. The process resulted in a succinic acid titre of 42.9 g/l, under optimised conditions (pH 5.0, 36 °C temperature and 39.7 FPU/g cellulase loading).

A study reported the effect of biotin on succinic acid production by *Actinobacillus succinogenes* (Xi et al. 2012). Many carboxylation and decarboxylation pathways require biotin as a necessary co-factor. Being an important co-factor for carboxylase enzymes, it ultimately has an indispensable function in the metabolic pathways concerned with carbohydrates, lipids and proteins. Additionally, biotin is known to considerably influence the synthesis of succinic acid and decrease the concentrations of by-products. Xi et al. (2012) reported the production of 45.2 g/l of succinic acid, with 90.4% yield by *A. succinogenes* in a 3-l bioreactor under optimised concentration of biotin.

Authors have also reported the effect of carbon dioxide on succinic acid synthesis (Herselman et al. 2017). Carbon dioxide acts a co-substrate for the production of succinic acid in many organisms including *A. succinogenes*. The transport and supply of inorganic carbon is achieved by the diffusional transfer of carbon dioxide from the medium to the cell through the cell membrane. Hence, carbon dioxide utilisation by the cell is proportional to the solubilised carbon dioxide concentration in the liquid medium. In the course of fermentation, the concentration of carbon dioxide dissolved in the medium will reduce if the carbon dioxide utilisation rate by the cells is greater than the availability of carbon dioxide to the medium. Carbon dioxide can be provided to the medium either in form of gas or as a salt of carbonate or bicarbonate in the medium. Salts of carbonate like calcium carbonate and magnesium carbonate are commonly supplied to the medium as a carbon dioxide source. A study reported a comparison between carbon dioxide and magnesium carbonate as a source of CO_2 for the synthesis of succinic acid by *Actinobacillus succinogenes* (Zou et al. 2011). A maximum succinic acid titre of 61.92 g/l was achieved, when the soluble carbon dioxide titre was maintained at 159.22 mM by augmenting the medium with 40 g/l magnesium carbonate as the source of dissolved CO_2, in addition to 101.33 kPa of partial pressure maintained by gaseous CO_2. In the same

study, the supply of gaseous CO_2 was completely replaced by magnesium carbonate as the sole source of CO_2 for the medium. This resulted in a succinic acid titre of 56.1 g/l, which was 7.03% lesser than the titre achieved under the availability of both magnesium carbonate and gaseous carbon dioxide.

Different physicochemical factors have different degrees of influence on the final yield of biomass and/or the product. Therefore, optimisation or standardisation of the influential parameters becomes crucial for increasing the yield and throughput of the process.

15.5.3 Scale-Up Studies

Biological methods for succinic acid production have a great potential to substitute the chemical-based approaches. This potential is attributed to the advantages such as utilisation of renewable substrates, and the fixation of carbon dioxide in the process of fermentation, owing to the capnophilic nature of the commonly used host microorganisms. Nevertheless, biological approaches for fermentation are commonly constrained by low rates of conversion. This calls for scale-up by utilising bioreactors with large volumes to obtain high throughput from the process. Bioreactors provide an ambient environment with controlled temperature, pH, agitation, etc. for encouraging the host organism to yield high biomass and product titres. Accordingly, the operation and configuration of reactors hold a vital significance in controlling the reaction rates, operating conditions, mass transfer, etc., which in turn governs the entire process economics (Ferone et al. 2019).

Several studies have outlined the use of packed bed bioreactors for succinic acid fermentations. Packed bed bioreactors are well-known for their characteristics such as the potential to function at high dilution rates and prevention of washout of the cells, provision for the re-use of the packed bed (or biofilm) support, high biomass titre and high volumetric productivity (Ferone et al. 2018). Longanesi et al. (2018) reported succinic acid synthesis by *A. succinogenes* biofilms in a packed bed reactor (1-l) in repeated batch mode from cheese whey. This resulted in a biomass titre of 4 g/l and succinic acid productivity of 0.72 g succinic acid per litre per hour. Ferone et al. (2018) reported the synthesis of succinic acid by *A. succinogenes* in a packed bed biofilm bioreactor in a continuous mode of operation. Using this approach at the optimum dilution rate of 0.5 h^{-1}, succinic acid was synthesised at a titre of 43 g/l, with a productivity of 22 g/l/h.

Studies have also delineated the use of fibrous bed bioreactors comprising of a fibrous matrix for the immobilisation of cells. This kind of reactors offers several benefits such as high biomass and product titre and stability of the fibrous matrix for long-term usage. Also, the cells used for immobilisation can be recovered with ease and recycled for the subsequent batches of fermentation. This results in significant reduction in expenses related to biomass production and product recovery (Chen et al. 2017). A study reported the synthesis of succinic acid by *A. succinogenes* using wastewater released from dextran fermentation process. The production was carried out under repeated fed-batch mode, in a fibrous bed reactor comprising of the cotton

fibrous matrix for immobilising cells. This approach resulted in a succinic acid titre of 56.5 g/l, with a productivity of 1.28 g/l/h (Chen et al. 2017). Li et al. (2017) reported the synthesis of succinic acid by metabolically engineered *Y. lipolytica* strain using glycerol as feedstock. When this fermentation was carried out in an in situ fibrous bed reactor under fed-batch mode, the process resulted in the synthesis of 198.2 g/l succinic acid (Li et al. 2017).

The availability of carbon dioxide to the cells inside a bioreactor plays a vital role in shifting the metabolic flux towards the production of succinic acid. Also, an increase in carbon dioxide availability results in improved solubilisation in the fermentation medium. An increase in the partial pressure of carbon dioxide can lead to enhanced carbon dioxide availability in the fermentation media, which in turn has a considerable impact on the production of succinic acid. Amulya et al. (2020) demonstrated a high-pressure gas fermentation reactor for the synthesis of succinic acid by *Citrobacter amalonaticus* and evaluated the effect of varying carbon dioxide partial pressures on the same. Carbon dioxide partial pressure of 2 bar resulted in the highest succinic acid titre of 14.86 g/l, with productivity and yield of 0.36 g/l/h and 52.10%, respectively.

Authors have also demonstrated succinic acid production in hollow fibre bioreactor. Hollow fibre bioreactors are characterised by a high ratio of surface area to volume, ease of recovering the biomass from the stream of product, prevention of washout of cells, reduced risk of contamination, easy elimination of inhibitory and toxic wastes in a continuous manner and high product titre (Wee et al. 2002). Wee et al. (2002) reported the synthesis of succinic acid from fumarate by *Enterococcus faecalis* under the continuous mode of operation in a hollow fibre bioreactor. Using this approach, the highest productivity of succinic acid achieved was 17.1 g/l/h. Authors have also reported the application of stirred tank bioreactor for succinic acid synthesis (Zou et al. 2011; Hoefel et al. 2012).

The choice of a reactor for scaling up the product and biomass depends to a greater extent on the characteristics of the cells and their growth requirements. The selection of an appropriate reactor configuration and mode of operation can greatly influence the process' sustainability and economic feasibility.

15.5.4 Downstream Processing

The biological production of succinic acid is often accompanied by the generation of other unwanted by-products like ethanol, acetic acid and lactic acid. The type of by-product produced may vary based upon the kind of microorganism strain, substrate and/or method used for the fermentation. Separation of these by-products from succinic acid is crucial to improve the purity of the final product. Downstream purification also eliminates the chances of inhibition that may be caused by these by-products of succinic acid synthesis (Mancini et al. 2020).

Li et al. (2010a) reported a single-step crystallisation technique for the purification of succinic acid from *Actinobacillus succinogenes* fermentation broth consisting of acetic acid, formic acid, lactic acid and succinic acid. At a pH less than 2 and

temperature of 4 °C, crystallisation of succinic acid occurred with a purity of 90% and yield of 70% (Li et al. 2010a). As far as crystallisation is concerned, different factors such as crystallisation time and a number of stages of crystallisation hold vital importance in enhancing the yield and purity of the final product. Luthfi et al. (2020) reported 17% increment in succinic acid recovery with an increase in the duration of crystallisation by 3 h. Also, the incorporation of a three-stage crystallisation process, in the same study, enhanced the recovery of succinic acid to 84.8% from an initial value of 55%.

Lately, studies have reported advanced membrane separation techniques for the recovery of succinic acid. Jusoh et al. (2020) developed an emulsion liquid membrane technique using vegetable oil for the extraction of succinic acid. Under optimum conditions, this approach resulted in a 100% yield and 21-fold concentration of succinic acid from the fermentation broth. Emulsion liquid membrane technology is an efficient approach owing to several advantages, such as limited power requirement and consumption, easy handling and operation and high mass transfer surface area. Studies have also outlined the application of more than one membrane separation technique to improve purity of the target product. Khunnonkwao et al. (2018) reported a two-stage membrane separation approach comprising of nanofiltration and reverse osmosis for the extraction of succinic acid from the broth. This method resulted in a yield greater than 92% and 99.5% purity. Membrane-based purification techniques are quite attractive alternatives as they can simultaneously lower the quantity of undesired by-products and extract the desired product.

Another very commonly used method for the recovery of succinic acid is through precipitation. Precipitation can be carried out by adding CaO or $Ca(OH)_2$. In this method, the fermentation broth is augmented with calcium ions to precipitate out calcium succinate. The resulting precipitates are filtered out, and succinic acid is recovered from the calcium succinate complex by using sulphuric acid. Further purification can be carried out by using ion exchange or carbon absorption and followed by concentration of succinic acid through evaporation and crystallisation (Mancini et al. 2020). Similarly, precipitation can also be carried out by using $Mg(OH)_2$ or $MgCO_3$. The addition of the magnesium-based compound into the fermentation broth results in the formation of magnesium succinate precipitates. Subsequently, free succinic acid is derived from this precipitate by using hydrochloric acid (Sosa-Fernández and Velizarov 2018). Also, precipitation of succinic acid from the broth can be carried out by using ammonia. This method requires the addition of an ammonium cation-based salt to form di-ammonium succinate precipitates. Free succinic acid is recovered from this precipitate by using sulphuric acid (Sosa-Fernández and Velizarov 2018). Sosa-Fernández and Velizarov (2018) compared these three approaches for precipitation, namely, ammonia-, magnesium hydroxide- and calcium hydroxide-based methods for the extraction of succinic acid from fermentation broth. The maximum percentage of recovery of 84.3% was achieved using ammonia. However, magnesium hydroxide-based precipitation approach was found to be favourable in terms of sustainability and economic feasibility.

Succinic acid can also be recovered from fermentation broths by using salting-out extraction, which employs an extractant (organic solvent) and a salting-out reagent (salt) to recover a hydrophilic compound from an aqueous medium. Sun et al. (2014) reported an acetone (organic solvent) and ammonium sulphate (salt) system for the purification of succinic acid from fermentation broth. Succinic acid with a yield of 65% and purity of 97% was obtained by using the salting-out approach in combination with crystallisation (Sun et al. 2014). One major disadvantage associated with the salting-out method is that a major fraction of the salt gets deposited in the bottom phase, and the recovery and re-utilisation of this salt (for the next cycle of extraction) is difficult. To counter this problem, sugaring-out approaches (comprising of sugar and a solvent) can be used as an alternative. The main advantage of this approach is that the sugar-containing phase can be recycled and used as a medium for succinic acid production. Sun et al. (2019) reported a tert-butanol (organic solvent) and glucose (sugar) system for sugaring-out of succinic acid. Seventy-three percent yield and 98% purity of succinic acid were achieved by using the sugaring-out approach in combination with crystallisation.

15.6 Industrial Market Scenario for Bio-based Succinic Acid Production

Out of the three trillion global chemical markets, ~15% will be derived from biological sources by 2025 (Dessie et al. 2018a, b). The bio-based chemicals are expected to reach a market of US$23,976 million by 2025 exhibiting a compound annual growth rate (CAGR) of 16.16% (Chandel et al. 2020). The abundance of renewable bio-feedstocks, the surge in carbon footprints, volatility in fossil fuel prices and high production costs are the major drivers for the shift from petro-based to bio-based succinic acid especially for applications like pharma, food, cosmetics and lubricants. The environmental benefits due to the fixation of carbon dioxide and the use of sustainable feedstocks further make the bio-based process a more attractive route than the conventional petro-chemical pathway (Chandel et al. 2020; Mancini et al. 2020; Lu et al. 2021).

To fulfil the surging market demand and harbour environmental benefits, many companies have engaged in the industrial production of bio-based organic acids. Thus, during 2013–2020, the bio-based succinic acid was expected to exhibit CAGR of 45.6% reaching a market volume of 710.0 kilo tons by 2020 (https://www.alliedmarketresearch.com/bio-succinic-acid-market). However, the high production costs (2.94 $/kg) is still a bottleneck in achieving bio-based production at a commercial scale (Ioannidou et al. 2020). Currently, companies such as Myriant Technologies, BioAmber, Reverdia and Succinity are the leading producers for the commercial bio-based synthesis of succinic acid. The bio-based production output varying from 10,000 to 30,000 tons/year was supplied to the global markets by these four pioneering companies (Jiang et al. 2017).

The selection of microbial strains is one of the critical parameters affecting the bio-based route as these producers' performance influences the cost, choice of

feedstock, the workflow of the downstream purification process as well as the overall productivity of bioprocess. Most of these companies employ synthetic and molecular interventions to create robust strains for functionality in harsh operative conditions. Reverdia Inc. employs an engineered yeast (*S. cerevisiae*) to work for succinic acid production even at pH 3, which overcomes the neutralisation step challenges for organic acids (Chandel et al. 2020). Similarly, an engineered *E. coli* strain is employed by Myriant. An evolved strain (*Pichia kudriavzevii* 13723) used by BioAmber produces succinic acid with a yield of 0.69 mol/mol glucose. The metabolic engineering tool was used by Succinity to produce a yield of 0.75 mol/mol glucose by *Basfia succiniciproducens*. This engineered strain could also yield succinic acid at 1.7 mol/mol by utilising glycerol and maltose effectively (Ferone et al. 2019).

Though these companies are producing succinic acid for specialised markets but to increase their outreach, the operating and capital costs need to be drastically reduced to achieve a production cost of 1 $/kg of acid (Ferone et al. 2019). Thus, to come up with an economically feasible technology for bio-based succinic acid synthesis, the following aspects need to be addressed majorly, i.e. utilisation of cheap feedstocks, engineering of robust strains or co-cultivating strains for consolidated bioprocessing, efficient operativity of bioreactors and simple downstream processing. A comprehensive perspective on suitability and operativity of different modes of bioreactor, viz. continuous, batch and fed-batch with free or immobilised biocatalysts for succinic acid production, has been highlighted by Ferone et al. (2019). Summarily, exploitation of novel microbial cell factories to engineer robust strains, waste feedstock utilisation as cheap substrates, optimised bioreactor conditions and downstream processing strategies will be required to achieve the commercial synthesis of bio-based succinic acid at competitive costs.

15.7 Existing Technological Challenges

Although research corresponding to the synthesis of bio-succinic acid has gained significant momentum in recent times, it is still plagued by various drawbacks that need to be addressed and challenges that need to be overcome for the successful commercialisation of this process in the long run. Various considerations relative to economic factors, sustainability, production and purification, genetic engineering of native strains, downstream processing, etc. have to be kept in mind for developing a holistic process for succinic acid production. The key challenges as of now have been discussed subsequently.

15.7.1 Environmental and Economic Impact

Depleting fossil-fuel-based energy resources, the rising price of crude oil supplies and the associated environmental damage caused due to their use have led to a search

for a cleaner, cheaper and, overall, more sustainable technology for the production of commercially important products.

Significant efforts have been made to switch from petroleum-based feedstocks to biomass-based feedstocks to make the production process more sustainable. However, it has been observed that biomass as the primary feedstock does not necessarily correspond to improved sustainability. In fact, in the absence of heat integration, the economic and environmental impact of alternative technologies was found to be higher than that of petroleum-based production processes (Morales et al. 2016). This is primarily because a significant amount of energy is expended in the separation of fermentation broth from the succinic acid produced, in bio-based processes. Current downstream processes thus exhibit a heavy dependence on energy consumption.

15.7.2 Low Yield and Purity

Another problem associated with using biological systems is the low product yield. The product concentration of succinic acid in the fermentation broth of bio-based production processes is comparatively lower than that of the petrochemical-based production processes. Thus, the net product yield is much lower when we use biological systems, offering a major disadvantage for the commercialisation of such technology (Cheng et al. 2012a, b). Even though continuous culture systems and fed-batch systems produce higher rates and titres, batch cultures are used for succinic acid production owing to the ease of operation.

The purity of the succinic acid produced is also compromised due to the presence of various other components in the fermentation broth. These include media components, inhibitory and non-inhibitory by-products, cellular intermediates, salts, extraction agents, other acids, etc. As a result, multi-step extraction processes have to be carried out to obtain succinic acid of high purity. This, in turn, increases the energy demand for the process while subsequently multiplying the net capital cost of production.

15.7.3 Product Inhibition

A key challenge associated with microbial fermentation is product toxicity to the cells. In certain cases, the toxicity of the organic acid produced can affect the economic viability of the fermentation process. Apart from the product of interest, there might be other by-products that may also contribute towards toxicity to the cells. Thus, there is a need for an organism that can not only produce the organic acid but also tolerate the product toxicity (Xiao et al. 2014).

15.7.4 Genetic Engineering of the Organism

For ensuring the economic viability of the biorefinery operations, it is important to identify cheap substrates, like lignocellulosic hydrolysates. The challenge lies in developing a strain that has the ability to utilise multiple sugars as well as tolerate the inhibitors present in the media. To understand and utilise the potential of such an organism, it is essential to map out its genome sequence in order to facilitate the identification of its metabolic pathways and conduct genetic engineering studies. The three major fermentation pathways for the synthesis of succinic acid include the oxidative TCA cycle, the glyoxylate pathway and the reductive TCA cycle, amongst which the last one produces the highest theoretical yield with glucose as a substrate. However, the current roadblock lies in the fact that there is a net deficit in NADH (reducing power) that needs to be addressed (Xiao et al. 2014).

15.7.5 Downstream Processing

More than half of the total cost of succinic acid production from microbial systems can be attributed to the separation of fermentation broth from succinic acid (Cheng et al. 2012a, b). The complication in separation is magnified due to the presence of various other components that make product recovery a cumbersome and expensive process. There are various methods by the means of which downstream processing is carried out. However, each of these come with their own set of disadvantages as follows:

1. **Ion exchange**: Ion-exchange method for the purification of succinic acid requires expensive resins which can add to the overall production cost. The selectivity of the resin is another factor that can influence the purity of the recovered succinic acid. Resins with low selectivity lead to lesser product purity and yield. The maintenance of these resins can also prove to be difficult in the long run.
2. **Electrodialysis**: Succinic acid can also be synthesised by the method of electrodialysis. However, this process is energy-intensive and consumes a huge amount of electricity, making it neither practical nor sustainable in the long run. Besides, the investment and maintenance cost of electrodialysis equipment is high. The electrodialysis membrane itself is prone to fouling. This in turn increases the overall complexity and cost of production.
3. **Precipitation**: The major disadvantage of precipitation using calcium oxide or hydroxide is the production of a high amount of calcium sulphate as a by-product, which is unfit for commercial use due to its smell as well as colour impurities. This process also requires a huge amount of sulphuric acid, calcium oxide and calcium hydroxide which cannot be recycled or regenerated. Thus, this method is not feasible for the large-scale synthesis of succinic acid (Kurzrock and Weuster-Botz 2010).

4. **Ultrafiltration**: Ultrafiltration is generally required to separate the cell debris. This unit uses special membranes for the filtration process which again gives rise to the problem of membrane fouling and heightened maintenance costs.

15.8 Future Perspective and Conclusion

Considering the existing challenges of product toxicity and low yield, it has become imperative to direct significant efforts towards developing an engineered strain that is as robust as it is efficient. Development of a strain where the pathway for by-product formation such as acetate, organic acids, ethanol, etc. is blocked can itself help in making the production process simpler and more cost-effective by minimising product inhibition and reducing the steps required for product separation. With the help of proper pretreatment methods, succinic acid can also be produced via simultaneous saccharification and fermentation (SSF) from lignocellulosic biomass. This method can further simplify the production process by combining microbial fermentation and enzymatic hydrolysis in one single bioreactor, thus minimising the need of multiple reactor setups (Akhtar et al. 2014). It is also necessary to develop a more convenient and economical downstream processing system to efficiently purify succinic acid. Methods such as single reactive extraction, vacuum distillation and crystallisation are thus currently being explored to obtain product purity as high as 99.5% (Akhtar et al. 2014). With the combination of improved strains, efficient downstream processes, cheaper substrates and effective technology, it is possible to envisage the commercialisation of clean and green technology for the synthesis of succinic acid. Owing to the efforts to shift towards a bio-based production method and attempts to alleviate the economic and ecological burden, the future presents a lot of hope for the production of this essential chemical.

Acknowledgement Gargi Goswami is thankful to her mentor, Prof. Debasish Das, Department of Biosciences and Biotechnology, Indian Institute of Technology Guwahati, for his constant guidance and support.

Competing Interests The authors state that the work was carried out without the existence of any financial or commercial associations that could be interpreted as a probable conflict of interest.

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Biomass, Bioenergy, and Biofuels

16

Anil Kumar Verma, Dixita Chettri, and Ashwani Kumar Verma

Abstract

The greatest challenge for humanity is the continuous provision of a sustainable source of energy while considering the environmental concerns of global climate change. These factors, along with the rising prices of fossil fuels, require research into various sources for the production of environmentally friendly renewable energy. Biomass has emerged as a key source in the contribution of renewable energy to meet future energy needs in the form of biofuels. It is a potential candidate for the production of electricity, heat, and transport fuels. The proper management of bioenergy will ensure energy security in the future and reduction of environmental pollution and realize the potential of organic waste, for economic and social development. The chapter gives an insight into the potential of biomass and technologies used for its conversion into bioenergy. The newly found use of algae and microbial cells as fuel has also been discussed.

Keywords

Biomass · Bioethanol · Biofuels · Bioenergy · Microalgae · Fuel cells

Abbreviations

ABE	Acetone-butanol-ethanol
AD	Anaerobic digestion
CHP	Combined heat and power
DMC	Direct microbial conversion

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_16

EU	European Union
MSW	Municipal solid wastes
PUFA	Polyunsaturated fatty acid
SSF	Solid state fermentation

16.1 Introduction

The use of biomass for various applications from food to feed as well as the generation of biofuels and biorefinery products has been under discussion since the last century (Garba 2020; Kumar and Verma 2021a). The energy crisis faced by the world since the 1970s has led several countries to shift their focus on the use of biomass for generating biofuels. Although various technological advances to reduce fossil fuel prices slowed down the development of biomass-to-biofuel conversion approaches for quite a long time, however, the ever-increasing fuel demand, fluctuating prices, limited supply of fossil-based fuels, and the emission of greenhouse gases and other harmful gases leading to global warming and environmental pollution have remotivated research into the development of biomass-based bioenergy(Lee et al. 2019; Kumar et al. 2020a; Kumar and Verma 2021b).

Biomass consists of solar energy along with CO_2 stored in the form of chemical energy (carbohydrate) via the process of photosynthesis. The trapped carbon can be released along with energy generation that makes biomass a potential renewable energy source (Sansaniwal et al. 2017; Chaturvedi and Verma 2013). The energy generated from biomass by using fuels is termed bioenergy. It is a renewable form of energy and can be harvested to meet global energy demand. Biomass can be used directly via combustion for the generation of thermal energy or can be converted through various technological innovations to generate different types of biofuels that can be used for various applications (Kumar et al. 2020b). Since the production of bioenergy from biomass has the advantage of providing sustainable energy while having additional benefits to the environment, the study of biomass potential and various aspects that have influenced its implication along with different technologies for biomass conversion is necessary (Long et al. 2013).

The energy contribution of biomass is already more than 90% in the rural areas of developing nations and is expected to be the leading energy source in the near future with a 10%–15% share globally (Bhavanam and Sastry 2011; Pathak et al. 2013). The chapter describes the types of biomass sources, key factors, technologies, and commonly used industrial biofuels.

16.2 Biomass

Biomass being a renewable source of energy has been considered a prospective source to be used as a feedstock for the generation of a sustainable form of energy to meet the present energy demand while providing security to future energy requirements. The application of biomass for energy generation dates back to the traditional use of firewood to generate thermal energy (Lee et al. 2019). For industrial applications, different range of feedstock can be used for biofuel production (Fig. 16.1).

Biofuels produced from edible food crops such as corn, sugarcane, sunflower, etc. are termed the first-generation biofuels, while lignocellulosic biomass such as switchgrass, straw, jatropha, etc. are used for the production of the second generation of biofuels (Naik et al. 2010; Sims et al. 2010). Recently algae are also being explored for the fabrication of the third generation of biofuels (Fig. 16.2) (Chowdhury et al. 2019).

16.2.1 Factors Affecting Resource Potentials

The biomass, due to its complex nature, makes it very obscure to estimate its potential as bioenergy with different literature showing zero technical to 1500 EJ of theoretical potential of the biomass for energy generation (Edenhofer et al. 2011). These differences depend on the assumptions of different scenarios such as plant type, yield, available area, and the methodologies used. Though different studies have been conducted for the estimation of the biomass to bioenergy potential, none of these studies include all the factors that could have an impact on the biomass potential. The biomass diversity, availability, and demand, water requirements, type and availability of land areas, superfluousness of food, competition with the other sectors for the resources in use, etc. are some factors that influence the potential of biomass for its use for generating bioenergy (Fig. 16.3) (Dornburg et al. 2010).

The type of crop, agricultural administration, history of a previous application for land under consideration, etc. directly affect the diversity of crops, which also affects

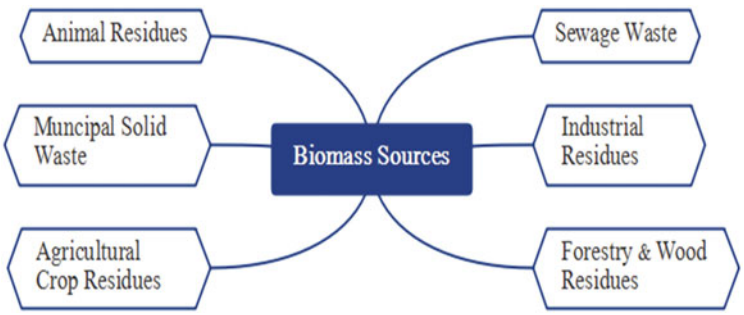

Fig. 16.1 The potential sources of biomass available for energy generation

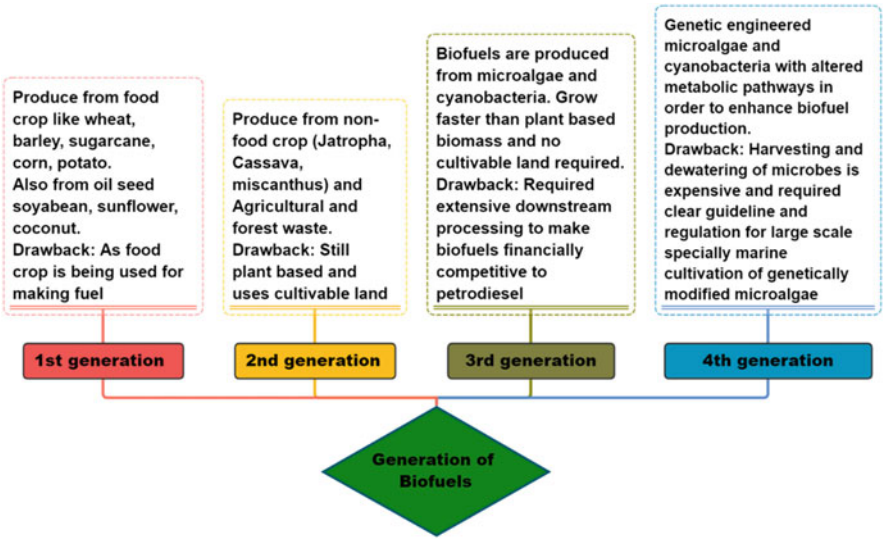

Fig. 16.2 Classification of biofuels based on biomass source

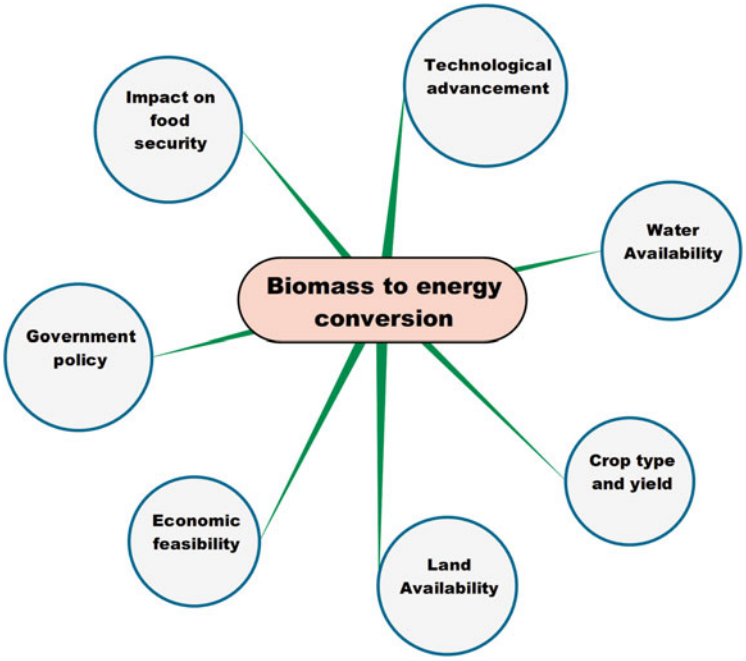

Fig. 16.3 Different factors that have an influence on biomass potential as a bioenergy source

the biomass potential. Furthermore, lower biomass demand for power generation due to its competition with other forms of renewable energy is another important factor. The demand for biomass is also dependent on the development of technologies to generate these alternate energy sources. Although various bio-based chemicals and biomaterials such as wood and fiber may have increasing biomass demand, they are not considered for biomass potential. To add to this, increasing food demand and the rising price of agricultural commodities have created a huge competition between the growing agricultural market and the use of bioenergy (Dornburg et al. 2010). It is estimated that if laws are enforced for the blending of fossil fuels and biofuels for transportation purposes, the price of the biofuel will upsurge by 10% using the first-generation biofuel crops (Banse et al. 2011). The use of cereals, sugarcane, oilseeds, etc. in biofuel production collides with food supply, while the use of pasture and grasses builds competition for limited resources such as land and water. The growing population shows a trend of increasing water demand for domestic and agricultural practices from 60 to 220% by 2050. Changing pattern of rainfall and increased rate of evapotranspiration due to climatic change all add up the scarcity of water for its use on bioenergy crop production. However, proper planning for the efficient use of water can provide favorable opportunities for biomass production for bioenergy production. Finally, an important factor to consider for biomass potential is its yield. The development of an efficient agricultural system with the application of modern technologies other than crop type can have a positive impact on biomass yield, yet successful implementation of these technologies in developing countries is an overwhelming task. However, the data regarding biomass potential are based on the use of perennial crops that usually have yields higher than the annual crops. Therefore, there are doubts about the reliability of these data while using annual biomass crops (Dornburg et al. 2010).

16.2.2 Feedstock Conversion Technologies

For the utilization of biomass potential, numerous conversion technologies are employed for generating energy in different forms. The type of conversion technology depends on biomass type, its characteristics, and quantity available, project specificity, end-use requirements or energy form required, economic and environmental policies, etc. with the most decision based on the form of energy required and pathway for its generation (McKendry 2002).

There are three main types of conversion technologies used for the generation of bioenergy from biomass to bioenergy: biochemical, thermochemical, and physiochemical with each type further divided into various processes for generating different energy types (Adams et al. 2018). Anaerobic digestion (AD) and fermentation are grouped under the biochemical technology of feedstock conversion, where AD is carried out by microorganisms for the conversion of organic waste to produce gaseous biofuel, i.e., biogas, whereas fermentation requires pretreatment and saccharification for the release of simple sugar molecules which are then fermented to produce liquid biofuel (ethanol). AD is more economical than fermentation as the

alcohol produced at the end of fermentation is diluted and thus requires an additional step of distillation (Deublein and Steinhauser 2011). Photobiological reactions are also being explored for the generation of biogas (biohydrogen) via conversion of biomass using the phototrophic organisms (Lee et al. 2019). Organic compounds are decomposed using high-temperature treatment to bring about their chemical conversion into biochar or to produce a liquid or gaseous biofuel in the thermochemical technology (Goyal et al. 2008). Combustion, pyrolysis, liquefication, and gasification are the options available under this technology. Combustion is the most common process used for the generation of heat and electricity (Kataki et al. 2015). In all thermochemical processes, pyrolysis is the initial stage as it brings about a chemical reaction in the absence of oxygen to produce all three forms of energy, i.e., solid, liquid, and gas (Patel et al. 2016). Physiochemical technology is used for the extraction of oil from the seed of different biomass such as linseed, which is also known as mechanical extraction. The oil is further esterified to generate biodiesel, which is used as a transportation fuel (Adams et al. 2018).

Faster reaction time, the ability to bring about the decomposition of even the recalcitrant biomass, and higher efficiency of the thermochemical process compared to other technology make them a popular option as feedstock conversion technology. Moreover, the energy released can be released in any form for various applications (Adams et al. 2018). Moreover, the readily available infrastructure, low water requirement, and the ability to use plastic waste for energy generation make thermal technology a widely used method (Uzoejinwa et al. 2018).

16.3 Bioenergy Production

The biomass can be used for the production of different biofuels, i.e., solid, liquid, as well as gaseous biofuels. Gaseous biofuel is biogas, whereas solid biofuels are sawdust, briquettes, straw, etc., while bioethanol, biodiesel, and biomethanol are liquid biofuels. Of these, liquid biofuels have higher demand as transportation fuels with biodiesel and bioethanol being the only biofuel used in the European nations (76 and 20%) (Brodziński et al. 2014).

16.3.1 Bioethanol

Ethanol generated using the renewable plant and microbial biomass as its substrate is termed bioethanol or good ethanol. It is an environmentally friendly and renewable source of energy (Johnston 2008). The production and usage of bioethanol reduces the energy dependency on fossil-based fuels and reduced the emission of greenhouse gases. Bioethanol is the most extensively used among liquid biofuels and is produced via the fermentation of sugar molecules from different sources. Different crops and agro-wastes can be used as feedstock for bioethanol production, such as sugarcane and corn, rice straw, switchgrass, pulpwood, as well as food waste and municipal solid wastes (MSW) (Demirbas and Demirbas 2010).

The use of cellulosic material for ethanol production requires pretreatment steps followed by saccharification, fermentation, and finally distillation for ethanol separation (Nigam and Singh 2011). Pretreatment is a necessary step for making cellulose molecules trapped in the hemicellulose and lignin components of the biomass accessible. Further saccharification of the cellulose and hemicellulose polysaccharides will convert them into simple fermentable sugars (Demirbas and Demirbas 2010).

Different technological advancement in ethanol production has been investigated to improve each step with *S. cerevisiae* as the most common organism for the fermentation step. Further technologies with combined saccharification and fermentation steps are being developed for ethanol production using lignocellulose. This SSF technology has the advantage of a high hydrolysis rate resulting in a higher yield at a shorter duration. Similarly, direct microbial conversion technology, i.e., DMC, involves single-step production, hydrolysis, and fermentation of cellulose that makes the overall process economical (Vasić et al. 2021).

Bioethanol finds its application in different industries such as cosmetic and alcoholic beverage production. However, the main studied application of bioethanol in the current scenario is bioenergy production. Bioethanol is mostly used as a transportation fuel with cars run entirely on pure ethanol or "gasohol," i.e., a blend of gasoline with ethanol (Bielski et al. 2015). The ethanol used in blending needs to be anhydrous with no engine modification required for its use as a blend. It enhances the octane rating of the fuel and at the same time reduces the amount of pollutants generated in unleaded gasoline (Demirbas and Demirbas 2010). The oxygen content of bioethanol is approximately 35%, while a low level of nitrous oxide is released in the environment. In the European nations, 5% blend of ethanol can be used with petrol without engine modification according to the EU quality standard EN 228 along with the vehicle warranty, while engine modification is required for using higher bioethanol percentage (Brodziński et al. 2014). Other countries such as the USA, China, Brazil, and Canada are also engaged in bioethanol production and use, with the highest production being shown by the USA which contributes more than 50% to the total global ethanol production followed by Brazil with 27% (Zabed et al. 2017; Vasić et al. 2021).

Though new technologies have greatly increased ethanol production, there are opportunities to increase the efficiency of the pretreatment processes of the cheap lignocellulosic biomass substrate, optimization of different components required for increased production, and enhancing tolerance and stability of the organisms used to make the overall process economical for wide-scale commercial applications (Nigam and Singh 2011).

16.3.2 Biobutanol

Butanol is a four-carbon alcohol that belongs to the same category as ethanol and methanol which are used as biofuel. Four isomers of butanol are present, namely, *n*-butanol, 2-butanol, iso-butanol, and tert-butanol, based on the orientation of the

carbon atoms which either form a straight chain or branched structure and the corresponding position of –OH (Zheng et al. 2015). Though these isomers have different properties, the energy generation and application in gasoline blending and combustion are identical. However, the production method of these isomers varies (Jin et al. 2011; Ramey 2004). *n*-Butanol is the commonly generated end product of the fermentation process with the fermentation technology being referred to as acetone-butanol-ethanol (ABE) fermentation as these three compounds are generated as the end product in a 3:6:1 ratio. The petrochemical-based product, tert-butanol, cannot be synthesized using biological methods, while 2-butanol production involves a two-step process, the first step being the bacterial fermentation of sugar to obtain an intermediate product, followed by chemical modification of the intermediate to 2-butanol directly in the fermentation medium. Iso-butanol is produced in a small amount by the yeast during the process of winemaking; however, large-scale production of iso-butanol is slowly being considered with different companies showing an increased interest in its production considering the issues with the production of *n*-butanol. Though commercial production of *n*-butanol goes back a long way, the complexity of the production process, toxic nature, and difficulty in recovery and purification make the overall process costly (Nigam and Singh 2011).

The fluctuation in the price of crude oil and achievement in bioethanol production has led to increased interest in the use of alcohol as a biofuel with the development in biobutanol production also being considered (Kumar et al. 2012). The same feedstock used for bioethanol generation can also be applied for butanol production, providing opportunities for the farmers as well as expanding the biofuel market since it is used alone or in synergy with ethanol for the preparation of gasoline blend. The higher hydrogen and carbon content of butanol makes blending with gasoline easier and has higher energy compared to ethanol. Butanol with a value of 85% can be used in gasoline blend to be used as fuel without modification in the car engines. The lower evaporation rate compared to gasoline and ethanol and emission of less volatile organic compounds make it an easy and safe alternative as biofuel. The lower corrosive nature makes transport and distribution of butanol easier using the system (Nigam and Singh 2011). Butanol also finds its application as a solvent and industrial cleaners other than acting as a fuel additive (Jin et al. 2011). However, the success of butanol production depends on technological advancement in the ABE fermentation technology. High pretreatment and recovery cost, the high toxicity of butanol to the fermenting microorganisms and end-product inhibition, and development of efficient strain for the fermentation process are the challenges to be addressed in this regard (Veza et al. 2021).

16.3.3 Biohydrogen

Hydrogen is another source of renewable energy with the potential for use as an alternative to conventional energy sources. It has numerous applications from use in fuel cells for generating electricity, fertilizer, and methanol production; in oil

refineries for removal of impurities; as a reducing agent, hydrogenating agent, or rocket engine fuel; and in cryogenics, pharmaceuticals, etc. Also, it is carbon-neutral energy with an energy yield 2.75 times higher than fossil fuels (Singh and Mahapatra 2019).

Though there are different methods for hydrogen production such as the electrolysis of water, fossil fuels, and natural gas, the commonly used method of hydrocarbon reformation generates carbon monoxide gas as a byproduct, which has environmental consequences. The thermochemical technologies used for biomass conversion to hydrogen also produce different toxic substances; thus the biological process of biomass conversion to biohydrogen is gaining attention (Mona et al. 2020). Biohydrogen production using photosynthetic organisms with zero pollution is one of the most efficient approaches with sunlight, water, and minimal nutrient requirements, and further technological advancement can provide a platform for commercial production of biohydrogen as renewable energy sources (Rodionova et al. 2017). The different species of bacteria, cyanobacteria, green algae, and plants produce biohydrogen along with oxygen via photosynthesis (Stevens 2001). Dark fermentation is another biological process where anaerobic fermentative bacteria are used for the conversion of sugar substrate to release hydrogen along with organic acid byproducts (Sen et al. 2008). This method of hydrogen production though has a higher yield and low concentration of hydrogen; however, it makes it uneconomical as a purification needs to be added for its use in fuel cells. Furthermore, the hydrogen yield depends on the fermentation pathway with 4 mol or 2 mol of H_2/mol of glucose being generated depending on whether acetate or butyrate is produced along with it (Wang et al. 2003). Photo-fermentation is another biological process in which photosynthetic organisms such as *Rhodopseudomonas palustris*, *R. capsulata*, *R. sphaeroides*, and *Rhodospirillum rubrum* use the energy from light for the anaerobic conversion of organic molecules to release hydrogen along with carbon dioxide (Basak and Das 2007).

Though biohydrogen can be used as a valuable and renewable source of energy, for economic viability and eco-friendly production, the use of cheap biomass for fermentation by the microorganism is preferred. Thus, studies focused on technological advancement in this area can enable the industrial production of biohydrogen using biomass and microorganisms (Saratale et al. 2019). The problems of storage and transportation are some other challenges that need to be exploited to make its commercial application feasible (Srivastava et al. 2020).

16.3.4 Biogas

Biogas is a gaseous alternate biofuel that is produced via the process of AD by microorganisms using different types of organic matter. The composition of biogas mainly consists of two gases, i.e., methane (CH_4) and CO_2 at 60% and 40%, respectively, with the presence of traces of other substances such as H_2S, siloxanes, NH_3, and water (Chaemchuen et al. 2016; IEA Bioenergy Task 37 2018). Various organic wastes such as agricultural residues, food scraps, municipal solid waste, and

industrial waste rich in organic compounds, etc. can be used for biogas production (Hanifzadeh et al. 2017). The use of these organic compounds helps to meet environmental guidelines while managing waste and providing cheaper or in some cases negative cost of substrate for generating renewable energy. The energy crops can also be used for generating energy with reduced emission of greenhouse gases (Zhu et al. 2019). Biogas can be produced in sewage and wastewater treatment plants, agricultural waste digestion plants, landfills, etc. through the action of mesophilic and thermophilic microorganisms (Chaemchuen et al. 2016). Furthermore, the residue generation at the end of AD can be used as fertilizer, thus making biogas a promising alternative biofuel generated using biomass (Palop et al. 2010).

Biogas can be used for the sole purpose of generation of heat as well as in combined heat and power (CHP) plants, where it can be used for generating electricity along with heat. The high content of CO_2 is an issue in the industrial utilization of biogas (Palop et al. 2010). The separation of CO_2 from biogas is essential to improve its calorific value. In addition, when biogas is used as a fuel, the purification of biogas is crucial as the contaminants present in the biogas can cause damage to the device and cause emission of undesirable compounds (Chaemchuen et al. 2016). The removal of CO_2 and other substances via purification generates an upgraded version of biogas known as biomethane, which has properties similar to fossil fuels and can be used directly with the existing transportation and distribution facilities (Zhu et al. 2019). Biomethane has received significantly increasing attention in the recent past in the context of renewable energy, with the number of biomethane plants rising from 187 to 465 within a span of 4 years (2011–2015) in Europe alone with a market significance of 90% globally (Cucchiella et al. 2017).

The development of technologies for the purification and cleaning of biogas is the key bottleneck for the exploitation of biogas to its maximum potential. Since the composition of contaminants depends upon the substrate source, with the contamination of H_2S being of major environmental concern, the separation technologies need to be established accordingly. The materials used in the purification steps should have high stability and nonreactivity to these contaminants. Therefore, biotechnological advancement in the evolution of suitable technologies with economic feasibility, maximum efficiency, and low energy are future expectations for large-scale commercial application of biogas as biofuel (Chaemchuen et al. 2016).

16.4 Algal Biomass as Fuel Cells

While the use of various food crops as feedstock for biofuel generation has all the environmental benefits of reduced carbon-dioxide emission and continuous supply of energy for the growing population, their use, however, can pose a major challenge in meeting the food demand and ensuring food security globally. Further limited land and water resources, exploitation of agricultural land for maximum production of these feedstock crops via the use of chemicals and fertilizers, soil erosion, loss of crop biodiversity, etc. can have a negative impact on soil health. A further limitation

Table 16.1 Application of algal biomass for the production of different biofuels

Biofuel	Technique	Algal species	Reference
Bioethanol	Fermentation	*Tribonema* sp., *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*	Wang et al. (2014), Özçimen et al. (2015)
Biodiesel	Transesterification	*Oedogonium*, *Spirogyra*, *Scenedesmus* sp.	Hossain et al. (2008), Chen et al. (2012)
Biogas	Anaerobic digestion	*Botryococcus braunii*, *Nannochloropsis oculate*, *Macrocystis pyrifera*, *Euglena gracilis*	Vergara-Fernández et al. (2008), Mussgnug et al. (2010), Frigon et al. (2013), Buxy (2014), Ciudad et al. (2014)
Biohydrogen	Biophotolysis	*Chlamydomonas reinhardtii*, *Synechococcus elongatus*, *Anabaena variabilis*	Happe et al. (1994), Markov et al. (1997), Mathews and Wang (2009)
	Fermentation	*Ulva* sp., *Chlorella vulgaris*, *Dunaliella tertiolecta*, *Chlorococcum littorale*	Ueno et al. (1998), Carver et al. (2011), Margareta et al. (2020)
Bio-oil	Pyrolysis	*Chlorella protothecoides*, *Nannochloropsis* sp., *Microcystis aeruginosa*	Miao et al. (2004), Miao and Wu (2004), Pan et al. (2010)
	Hydrothermal liquefaction	*Chlorella pyrenoidosa*, *Scenedesmus*, *Spirulina*	Yu et al. (2011), Vardon et al. (2011, 2012)

of current technological advancement leading to the high cost of pretreatment methods and low yields makes the use of the lignocellulose uneconomical (John et al. 2011). To address these issues, the third generation of biofuels based on algal biomass is being introduced, which can serve as the best alternative for addressing the urgent demand for biofuels without compromising food security or the limitation of agricultural land (Subhadra and Edwards 2010) (Table 16.1).

Microalgal biofuels show characteristics similar to fossil fuels and have gained considerable attention in the past decade. Different species of algal are being explored for their capability to produce biofuels, especially bioethanol with increased investment in the research and development in this area by the different fuels-based companies (Kiran et al. 2014). The higher content of oil in their biomass makes the microalgae a potential substitute for crude oils (Mehariya et al. 2021). Other advantages of the algae over other feedstock crops include higher CO_2 sequestration (1.83 kg CO_2/kg of algal biomass); bioremediation of industrial, agricultural, and municipal wastewater via removal of chemicals; and heavy metals NH_4^+, NO_3, PO_4^{3-}, etc. The algae can be cultivated throughout the year and have the ability to thrive and grow under a low nutritional environment with no meddling with food security (Goswami et al. 2021). Their water requirements are lower than the other feedstock crops, and they can be cultivated in nonarable land with no requirement of fertilizer or pesticides. The fast growth rate and accumulation of

different neutral lipids for a higher yield of biodiesel are favorable for algal biomass-based biofuels (Goswami et al. 2021). Other important compounds such as PUFA, pigments, dyes, proteins, etc. can also be obtained from algal biomass (Kiran et al. 2014).

The algal biomass can be used for biofuel production via the conversion of algal metabolite into simple sugars. The algae assimilate a large amount of starch in their cell or, in some cases, other carbohydrates such as cellulose or laminarin, which can then be harvested and converted into simple sugars to be used for fermentation using efficient ethanol-producing organisms, similar to most biofuel generation technologies (Bhardwaj et al. 2020a; Agrawal et al. 2020). In the dark, the algae generate energy through the breakdown of stored carbohydrates, i.e., starch and glycogen via oxidative reaction. If the anaerobic condition is maintained during this time, the incomplete degradation of starch can lead to the generation of ethanol, H_2 gas, organic acids, etc. in varying proportions depending on the algal species. This process can be modified for these algae to operate in the form of a mini-factory for ethanol production via dark fermentation. Further attempts at the development of microalgae genetically engineered for direct ethanol production were also done (John et al. 2011; Chaturvedi et al. 2021).

16.5 Improving the Capabilities of Microbial Strains for Bioenergy Production

Improved conversion of plant biomass into various bioenergy sources could be achieved by implementing genetic engineering to construct relevant microbial strains with enhanced lignocellulosic degradation capabilities (Singhvi et al. 2014). Various genetic engineering methods can help to improve biochemical reaction rates to achieve maximum end-product production. Various methods such as heterologous expression of genes encoding plant biomass hydrolytic enzymes, expression of transporter proteins and carbon uptake pathways, expression of CO_2 fixation pathways, etc. in relevant microorganisms could be used to achieve improved bioenergy production. Some important approaches are discussed.

16.5.1 Consolidated Bioprocessing (CBP)

An innovative approach to effective bioenergy production is consolidated bioprocessing (CBP). In this approach, all three steps of biofuel production, enzyme production, and saccharification and fermentation are combined in the same reactor, where the pretreated plant biomass is efficiently converted into the desired product by the microbial consortium without the addition of saccharified enzymes (Kumar and Verma 2020a, b).

There are two strategies for CBP: native strategy and recombinant strategy. Native strategies include naturally hydrolytic enzymes producing microbial strains and improving biofuel production by using different approaches such as isolating

new strains for CBP and using different substrates (Salehi Jouzani and Taherzadeh 2015). Microbial candidates suitable for this strategy include hydrolytic enzyme-secreting bacteria and fungi and cellulosome-forming bacteria. Liu et al. investigated the ability of the native cellulolytic bacterium, *Clostridium thermocellum* DSM 1237, to produce bioethanol from lignocellulosic biomass. The industrial applicability of this bacterium was concluded by high rates of cellulose degradation and the ability to survive and grow at higher temperatures of 50–60 °C, both properties that are advantageous for industrial processes (Lamed and Bayer 1988). In this study, the strain *C. thermocellum* DSM 1237 was cultured in 3-L fermenter and anaerobic flasks, and the growth of the strain was evaluated under different cellular growth conditions such as different temperatures, different carbon sources (glucose, cellobiose, and xylose), and substrates and its ability to produce ethanol. The different fermentation substrates used for the evaluation included rice straw, corn straw, sugarcane bagasse (SCB), peroxide fortified alkali-treated SCB, and SCB treated with alkali. With 0.5% (weight/volume) cellobiose and an optimum temperature of 60 °C, the strain produced ethanol with a yield of 0.60 g/L at 0.80 g/g cell biomass. Utilization of alkali-treated sugar bagasse showed an increased yield of ethanol to about 0.68 g/L. The addition of enzymes such as xylanases and cellulases in the 3-L fermenter showed further improved yield of ethanol, i.e., 0.86 g/L, which is 83.3% of the theoretical yield. Thus, the integrated one-step hydrolysis and fermentation process and the on-site addition of lignocellulolytic enzymes, i.e., CBP, proved to be an effective approach to increase bioethanol production (Liu et al. 2020; Bhardwaj et al. 2020b). Second, the recombinant strategy involves engineering the ability to secrete hydrolytic enzymes in non-cellulolytic microorganisms and implementing these modified strains for enhanced production of bioenergy such as biofuels. Some commonly used microbial hosts for the recombinant strategy are bacteria such as *Escherichia coli*, *Lactobacillus lactis*, *Zymomonas mobilis*, *Bacillus subtilis*, etc. and yeasts such as *Saccharomyces cerevisiae*. *E. coli* FBR strains were constructed using ldh (lactate dehydrogenase) and pfl (pyruvate formate lyase) strains. The strains were transformed with pLOI297 plasmids containing the pet operon system. These recombinant strains of *E. coli* were able to produce ethanol from various substrates such as xylose, arabinose, and glucose. Strain FBR5 showed maximum ethanol production with 0.46–0.51 g/L yield (Dien et al. 2000). Xylose fermentation ability was introduced into *Z. mobilis* by insertion of four *E. coli*-derived xylose fermentation genes: *xylA* (encodes xylose isomerization), *xylB* (encodes xylulose kinase), *tktA* (encodes transketolase), and *tktB* (encodes transaldolase). The genes were introduced into the bacterium under the influence of two strong constitutive promoters: Glyceraldehyde 3-phosphate dehydrogenase and enolase promoters. The transformed strain thus produced, *Z. mobilis* CP4 (pZB5), showed efficient conversion of xylose to produce a high yield of ethanol of about 86% (Parker et al. 1995). Another important approach to increase bioenergy production under CBP is the overexpression of hydrolytic enzymes. Such overexpression of lignocellulolytic enzymes can be achieved by various techniques, such as increasing the copy number of genes, engineering the specific promoters, protease enzyme deficiency, etc. Yamada et al. showed increased production of

ethanol from *S. cerevisiae* by the technique of increasing the copy number of genes. When grown on substrates such as pretreated rice straw and cellulose treated with phosphoric acid, the transformed yeast showed ethanol production with yields of 7.7 g/L and 7.6 g/L, respectively (Yamada et al. 2010).

In addition to the advantages of the CBP approach, both strategies have their limitations. The native biomass-degrading microorganisms are usually wild type and are poorly characterized. Such organisms isolated with the desired CBP potential perform well below satisfactory levels of bioenergy production. In the recombinant strategy, setbacks include the undesirable effects arising from the co-expression of multiple genes, improper protein folding, variations in the corresponding expression levels of the different genes, inadequate fermentation, etc. These limitations could be efficiently addressed to make CBP an efficient approach for future biotechnological fields.

16.5.2 Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-Associated Proteins (Cas9)

Recent advances in the fields of genetic engineering have provided a plethora of techniques and tools to modify the physiological behavior of a relevant microorganism in the desired form and consolidate its applicability in the industrial fields. In the genetic engineering approach, the desired gene can be inserted, deleted, or regulated at a specific site of the chromosome of the host microorganism. There are two major types of genetic engineering: MEM engineering and REM engineering. MEM genetic engineering includes three methods zinc finger nucleases (ZFNs) (Miller et al. 2007), transcription activator-like effector nucleases (TALENs) (Joung and Sander 2013), and meganuclease system (Silva et al. 2011). REM genetic engineering includes CRISPR/Cas9 technology.

CRISPR/Cas9 technology was originally derived from the adaptive immune systems in bacteria and archaea. In the bacterial genome, this system contributes 40% of the endogenous adaptive immune defense and approximately 70% in members of archaea (Burstein et al. 2016). CRISPR/Cas9 provides defense in three stages: adaptation, expression, and interference against invading exogenous DNA. The invading DNA is fragmented into multiple fragments by Cas genes to produce protospacers. Such protospacer fragments are introduced into the CRISPR locus tandem array. In the second phase of defense, expression, the locus with the integrated spacer is transcribed to produce long precursor CRISPR RNA (pre-crRNA), which forms a complex with transactivating CRISPR RNA (tracrRNA) and Cas9 protein. The RNA hybrid is recognized by an RNA exonuclease enzyme, which converts the complex into a mature form. The mature form of the RNA hybrid combines with the Cas9 protein, which cuts the DNA to create double-strand breaks at the desired locations. Replacing the dual RNA hybrid with a specifically designed guide RNA, called sgRNA, forms the basis of CRISPR/Cas9-mediated gene manipulation to incorporate desired traits into host cells. The sgRNA attachment requires a protospacer adjacent motif (PAM) present

immediately downstream of the target DNA site. Generated DSBs are repaired via two repair pathways: homologous repair (HR) pathway and the nonhomologous end-joining repair (NHEJ) pathway. NHEJ can lead to gene insertion and deletion, while the HR pathway uses an exogenous DNA donor to recombine at the desired sites. Gene knockout and gene knocking introduced via DNA repair can be used for the addition or elimination of desirable traits within microorganisms.

CRISPR/Cas9 gene-editing technology has been applied to a variety of industrial microorganisms, including bacteria, yeasts, and fungi, to enhance their capabilities in the production of bioenergy, such as biofuels. This approach of genetic manipulation has been studied to install a number of desirable traits in the producing strains, such as:

1. Building increased tolerance to biofuels: different microorganisms exhibit differences in their biofuel tolerance. Several functional, genomic, and transcript profiling analyses of *S. cerevisiae* revealed the presence of genes controlling tolerance to ethanol (Lewis et al. 2010), suggesting an increase or decrease in ethanol tolerance following site-specific mutations at such genes. In *Z. mobilis*, ethanol tolerance was increased by a frameshift mutation in the gene encoding the enzyme NADH dehydrogenase (Ulaganathan et al. 2017).
2. Increased tolerance to various inhibitors: tolerance to inhibitors is important to prevent low yields due to the production of toxic compounds during pretreatment processes of lignocellulosic biomass. Ramos et al. developed increased tolerance to acetate in *S. cerevisiae*. The removal of a single amino acid from four different genes, GLS4, ADH3, SKS1, and ASG1, which were present at different sites, showed increased tolerance of the yeast cell to acetic acid (González-Ramos et al. 2016).
3. Enhancing the tolerance to temperatures: one amino acid alteration in NADH dehydrogenase enzyme and pyruvate kinase showed enhanced ethanol production and more thermotolerance in the bacterium *Z. mobilis* (Benjaphokee et al. 2012).

However, although equipped with several advantages, the recent technology of CRISPR/Cas9 provides certain limitations which include off-target effects, low efficiency of gene manipulation by HR, absolute dependence on the PAM site, as well as the challenges of the generation and the delivery of sgRNA. Such limitations need to be addressed in a more efficient way to make this technology applicable on industrial grounds (Table 16.2).

16.6 Conclusion and Prospects

Biomass and bioenergy have become a major global issue, with a remarkable increase in research and development and an expanding market for bioenergy. Biomass is a continuously available renewable energy resource, and its full potential for use as a bioenergy source has yet to be explored. It has the potential to contribute

Table 16.2 Strategies for improving the ability of microbial strains for bioenergy production

Consolidated bioprocessing (CBP)					
Native strategies	**Microorganisms**	**Method**	**The objective of the method**	**Yield**	**References**
	Thermoanaerobacterium sp.	Deletion of *ldhA* gene encoding lactate dehydrogenase enzyme	Improvement in hydrogen production	Hydrogen production increased by twofold	Shaw et al. (2008)
	Clostridium saccharoperbutylaceticum strain N_{1-4}	Hydrogenase gene (*hup*CBA) cluster downregulation using antisense RNA technology	Improvement in hydrogen production	Improvement in hydrogen evolving activity by 3.1-fold	Nakayama et al. (2008)
	Clostridium thermocellum CT2	Anaerobic single-step conversion of banana crop waste by co-culture fermentation of CT2 with *C. thermosaccharolyticum* and *Thermoanaerobacter ethanolicus* ATCC 31937 strains	Enhanced bioethanol production	A maximum ethanol yield of 22 g/L obtained on the substrate of 100 g/L concentration	Harish et al. (2010)
	Clostridium acetobutylicum	Inactivation of *hbd* gene essential in butyrate synthesis	Improvement in ethanol production	High ethanol yield of 0.38 g/g of glucose with a productivity of 0.5 g/L/h	Lehmann and Lütke-Eversloh (2011)
Recombinant strategies	*Clostridium cellulolyticum*	Heterologous expression of two genes: *Pdc* and *Adh* encoding pyruvate decarboxylase and alcohol dehydrogenase enzymes, respectively, and isolated from *Zymomonas mobilis*	Improvement in ethanol production	Improvement in ethanol production by 53%	Guedon et al. (2002)
	Escherichia coli	Heterologous expression of different genes: *thl*, *bcd*, *crt*, *etfA*, *etfB*, and *adhE* involved in butanol pathway and isolated from *C. acetobutylicum*	Production of biobutanol	Highest butanol titer by BUT2 strains reported was 1184 mg/L	Inui et al. (2008)
	Saccharomyces cerevisiae				

		Co-expression of endoglucanase enzyme from *Trichoderma reesei* and β-glucosidase from *Saccharomycopsis fibuligera*	Enhanced production of ethanol	Higher yield of ethanol with 1 g/L	Den Haan et al. (2007)
CRISPR/Cas9					
	Myceliophthora thermophila	Multiplex genome editing using CRISPR/Cas9 method. Efficient mutation of the imported gene amdS in the genome of the fungi	Production of strain with hyper-cellulase activity	Increase in lignocellulosic activities by 5- to 13-fold	Qian Liu et al. (2017)
	Chlamydomonas sp.	CRISPR/Cas9-mediated knockout of gene involved in lipid degradation	Improvement in lipid accumulation	6% increase of lipid accumulation in the mutant cell	Nguyen et al. (2020)
	Escherichia coli ASA02	CRISPR/Cas9-mediated integration of butanol synthetic cassettes after the elimination of gene involved in ethanol production	Production of *n*-butanol	Butanol production by 4.32 g/L	Abdelaal et al. (2019)

greatly to the global energy supply in the coming years. Although its commercial realization depends on its success in competing with fossil fuels and on environmental and agricultural policies, its share in the global energy market is steadily increasing. Various factors and available technologies for biofuel production are the most important considerations in selecting from the wide range of available biomass sources. The study of all these factors and technological advancements will further improve the biomass and bioenergy market.

Acknowledgments The authors would like to thank the Department of Microbiology, Sikkim University, for providing the computational infrastructure and central library facilities for procuring references.

Conflict of Interest Statement The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Biomethanation: Advancements for Upgrading Biomethane Using Biogas Technologies

17

Priti Khandubhai Mahla, Priya Chandulal Vithalani, and Nikhil Sumantray Bhatt

Abstract

The biological production of biodegradative derivatives has received attention among technocrats because of the attractiveness of renewability and sustainability. Urban industries have to realize the environmental impacts and costs of waste disposal, turning this problem into an economically sustainable initiative. Biogas production through anaerobic digestion can provide an efficient way to attain several objectives concerning energy, environmental, and waste management. Biogas contains CH_4 (60%) and CO_2 (40%) as its principal constituent. Excluding methane, other gases present in biogas are considered as contaminants, which act as a bottleneck for fuel efficiency. Removal of these impurities will increase the quality of biogas. The present work is a critical review that summarizes state-of-the-art technologies for biogas upgrading with particular attention to the emerging biological methanation processes. This chapter also provides future perspectives on strategies to overcome the challenges associated with biogas upgradation. Biogas offers a good substitution for fossil fuels, but still not a perfect solution for remediation of increasing global greenhouse gas emissions. Therefore, more research still needs to be conducted for developing a self-sustainable and economical approach for biomethane upgradation.

Keywords

Anaerobic digestion · Biogas upgrading · Biomethanation · Scrubbing · Feasibility

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_17

Abbreviations

BEC	Bioelectrochemical
CSTR	Continuous stirrer tank reactor
MEC	Microbial electrolysis cell
UASB	Upflow anaerobic sludge blanket reactor

17.1 Introduction

17.1.1 Historical Introduction

Methane (CH_4) containing flammable gas mixture produced via biomethanation process with the help of microorganisms is called biogas. History of biomethanation noted that in the early 1630s Jan Baptista Van Helmont stated that flammable gases can be generated from accumulated decayed organic matters. Several reports suggest the generation of flammable gas at the site of organic waste decomposition which can lead to accidents. In 1776, Alessandro Volta projected the direct relationship between organic matter quantity and amount of total gas produced. During 1804–1808, John Dalton and Humphrey Davy suggested that on burning of biogas the flame was generated due to the presence of methane gas in mixture and it is the major constituent of total gas (Tietjen 1975). The generation of biogas was reported by several groups, and the Dutch farmer Wouter Sluys was one among them. In 1875, he utilized methane generated during waste biomass decomposition for providing energy for the illumination system (McCarty 1982).

In India, Matunga Leper Asylum at Bombay (Mumbai) constructed the first anaerobic digestion system plant that could produce biogas using human waste. The biogas produced at the system was used to fulfill the illumination requirement (Khanal 2008). In 1900, at Mumbai then Bombay, attempts were made for the generation of biogas using manure as substrate; however the yield was not sufficient. In 1937, S.V. Desai (microbiologist) from the Indian Agricultural Research Institute (IARI) demonstrated the successful operation of a biodigester for many years. During the 1950s, an in-depth examination of several types of plant designs was performed . The very successful model was “GramaLaxmi III” constructed by a Gandhian worker from Gujarat named Joshbai Patel. The floating dome model was supported by Khadi and Village Industry Commission (KVIC). The biomethane production unit named as “Janata biogas plant” was established by “Gobar Gas Research Station” at Ajitmal, Uttar Pradesh, India. Janta biogas plant adopted drumless dome-shaped model from China. Several firms in Nepal developed similar system and design with the help of various groups from India and termed as Biogas Sector Partnership (BSP) (Paramaguru and Kannan 2019).

17.1.1.1 Microbiological History of Biogas

In 1868, for the first time Bechamp suggested that the microbial processes were responsible for methane formation occurring by decomposition of organic matters. During the 1890s, Omelianski suggested that microorganisms assisted in the production of acetic acid, butyric acid, and hydrogen during biofermentation. Also, he recommended that probably the reaction between carbon dioxide and hydrogen that results in the generation of methane was carried out by microbes. Later in 1910, Sohngen took the initiative to carry forward Omelianski's discoveries and explained that for the generation of acetic acid, carbon dioxide, and hydrogen there were 17 fermentation processes of organic substances via oxidation-reduction reactions and methane could be produced. Also, it was demonstrated that methane was produced due to reaction between carbon dioxide and hydrogen. Additionally, the decarboxylation of acetic acid can also result in the generation of methane (McCarty 1982).

17.1.2 Biomethanation Process

Methane can be produced by anaerobic digestion of organic complex materials through a multistage process that includes four stages (Kumar and Verma 2021). Figure 17.1 displays the process of biomethanation (Gashaw 2014).

17.1.2.1 Hydrolysis

The first stage of biomethanation is called hydrolysis and in this stage, organic polymers (biomass) are utilized by anaerobes to digest it and decompose complex materials into simpler forms. Complex materials like carbohydrates, fats, and protein can be decomposed into sugars, fatty acids, and amino acids. If there are any larger molecules left prior to hydrolysis that cannot be decomposed, then it would carry forward to the next stage of biomethanation.

17.1.2.2 Acidogenesis

In this stage fermentative microbes play a key role to ferment sugars, fatty acids, and amino acids into short-chain organic volatile fatty acids. It occurs due to butyric, lactic, propionic, and valeric acid production by native fermentative microbes.

17.1.2.3 Acetogenesis

Throughout the acetogenesis stage, carbon and energy source react and produce acetate. During acetogenesis, the product and energy generated during the previous stages are utilized for the formation of acetate. This new product could be utilized in the last stage of methane production.

17.1.2.4 Methanogenesis

The last stage of anaerobic digestion of biomethanation is methanogenesis to obtain methane. The product of the previous stage (acetate) is utilized here and results in the generation of methane, carbon dioxide, and hydrogen sulfide.

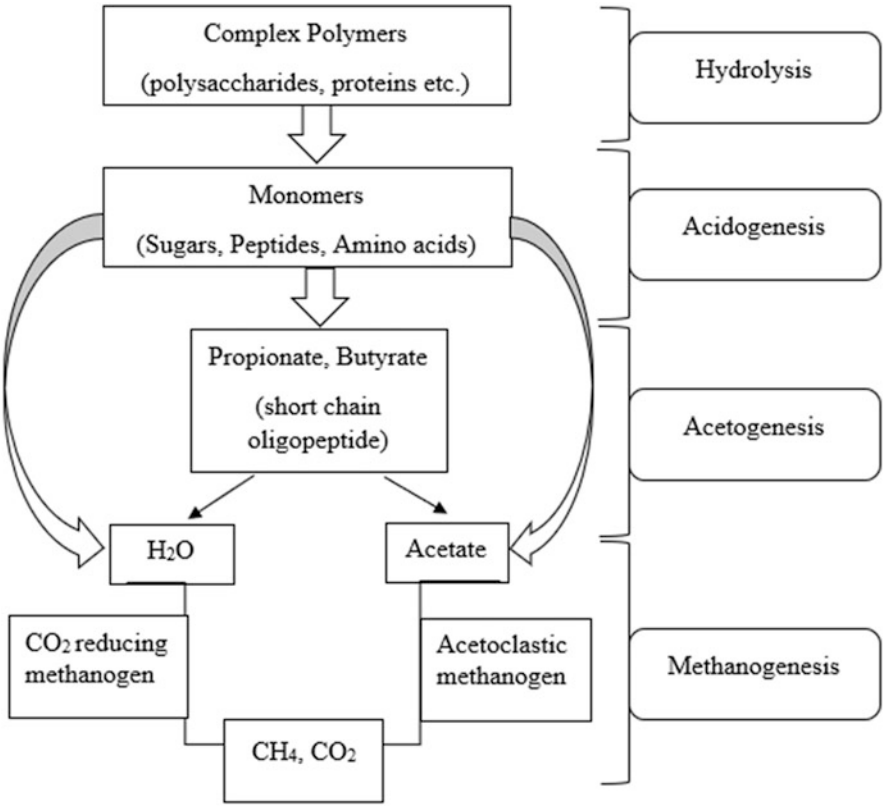

Fig. 17.1 Biomethanation process

17.1.3 Component of Biogas

Biogas can be produced utilizing different types of substrates and microorganisms, The production of biogas is controlled by several parameters such as physical condition, raw materials, and microbes used in the anaerobic digester system. Various reports suggest that biogas yield and quality (constituent/composition) greatly depends on the type of biomass used as substrates and methods adapted for biogas production. The raw biogas cannot be used directly for commercial utilization as along with methane several other components are present as well.. Table 17.1 contains a general range of components (Andriani et al. 2014).

17.2 Technologies for Biogas Upgradation

Biogas can be used in gas grid injections, fuel, heat, and electricity, provided it has high purity (free from impurities) and composition of methane is high in the mixture (Angelidaki et al. 2018). As the biomethanation process is carried out by a wide

Table 17.1 Component of biogas

Component	Content (%)
Methane (CH_4)	40–75
Carbon dioxide (CO_2)	15–60
Water (H_2O)	5–10
Hydrogen sulfide (H_2S)	0.005–2
Siloxanes	0–0.02
Halogenated hydrocarbons (VOC)	<0.6
Carbon monoxide (CO)	<0.6
Nitrogen (N_2) (can be)	0–2

variety of methanogenic microorganisms, several other value-added products formed along with methane. Biogas produced via anaerobic digestion consists of different constituent gases such as CO_2 and H_2S; thus its upgradation is mandatory for its commercial utilization. Therefore, several biogas purification processes or biogas upgradation techniques are developed for cleaning biogas to utilize it fruitfully. The application of biogas in different sectors (such as domestic and industrial) only depends on the quality of biogas and anaerobic digestion (Li 2012). If a local homemade biogas plant is constructed for biogas production to meet the domestic cooking requirement, then purity of biogas is not considered much but still high concentration of methane is required. However, for sophisticated applications such as running of engines, gas grinding, or instruments, high purity of biomethane is much needed, Biogas with high purity is suggested to be used globally for several industrial applications. Therefore, enhancement of the purity of biogas through several upgradation approaches such as physical, chemical, and biological methods was reported as shown in Fig. 17.2 (Adnan et al. 2019).

17.2.1 Physicochemical Upgradation

Various physical, chemical, and combination of both conventional upgradation technologies are in practice and are evolving day by day. Methods like chemical and water scrubbing by absorption, separation by membrane filtration, carbon molecular and activated carbon sieve, cryogenic separation, and pressure swing adsorption are in continuations for upgradation of biogas (Khan et al. 2017).

17.2.1.1 Chemical Absorption Using Amine/Alkaline Solutions

Amine solutions can be used by chemical scrubbers for upgradation process. Aqueous amine solutions, for example tri-ethanolamine, or mono-, di-ethanolamine, can bind with the CO_2 as well as H_2S molecules modified in the biogas. Thus, this can be a very advantageous method for removing CO_2 and H_2S (Kapoor et al. 2019). The structure of amine scrubbing systems contains two major units, an absorbent and stripper unit, as shown in Fig. 17.3. Amine/alkaline solution passes through the absorption column from the top in a counter-current manner while biogas with 1–2 bars pressure flows from the bottom of the tank. The exothermic chemical reaction is

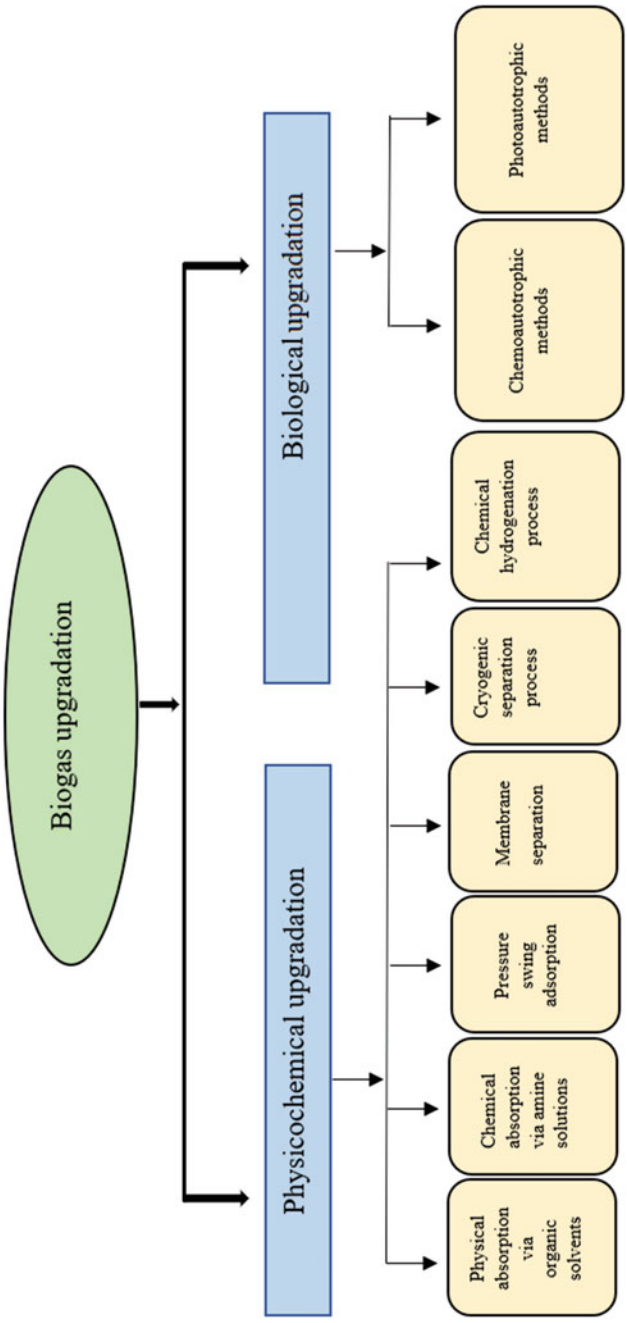

Fig. 17.2 Biogas upgradation methods

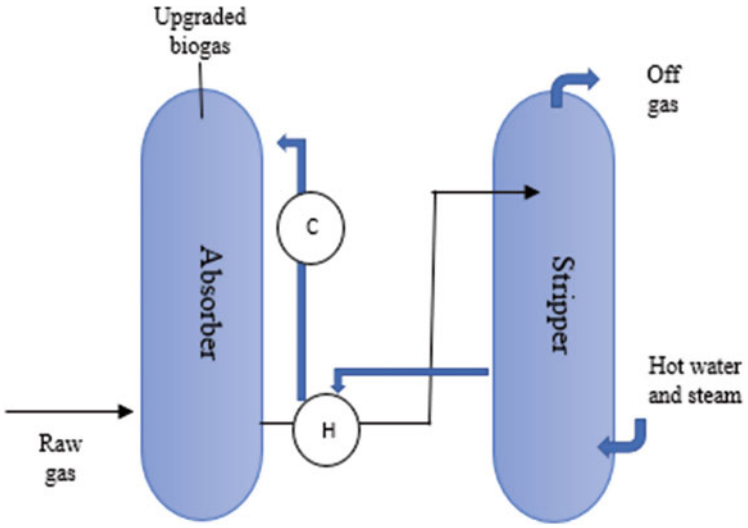

Fig. 17.3 Chemical absorption using amine/alkaline solutions

carried out there, and CO_2 and H_2S get bound into the solvent. Afterward, the stripping unit came into action, for regeneration of trapped CO_2 and H_2S-rich amine/alkaline solution is directed to the stripping unit. The stripping unit is attached with boiler with pressure bars of 1.5–3. Equipped boilers can be heated up to 120–160 °C. The heat applied in the process breaks down the chemical bonds which might be formed in the absorbent column. The heat also helps in the generation of vapor which later turns into fluid. At the last stage named as scrubbing, CO_2 and H_2S are trapped by recirculating the steam into condenser and stripper. This resulted in cooling of vapor for release of entrapped impurities (Kougias et al. 2010; Zhao et al. 2010). The aqueous alkaline salt solutions such as sodium, potassium, and calcium hydroxides react with CO_2 and H_2S and can be utilized for the chemical scrubbing technique instead of only amines (Baciocchi et al. 2012). Sodium hydroxide salts have more CO_2 absorption ability compared to mono-ethanolamine solution. Yoo et al. (2013) explained that to achieve 1 ton of CO_2 trapping, 1.39 tons of mono-ethanolamine would be required as a theoretical amount, while for the equivalent amount of CO_2 trapping only 0.9 tons of sodium hydroxide will be consumed. There are several demerits of chemical scrubbing like toxic nature of solvents that can be harmful to humans and the environment, substantial energy needed for the renewal of chemical salts as well as solutions, the evaporative loss, and the high cost of the amine solvents. Therefore, many researchers utilize aqueous alkaline salts rather than amines. In the chemical scrubbing technology, the final methane content in the output gas can reach up to 99% of purity due to the fact that the chemical reaction is strongly selective, and thus the methane loss can be lower than 0.1% (Angelidaki et al. 2018).

17.2.1.2 Physical Absorption via Organic/Water Solvents

This method is similar to water scrubbing principally, the major difference being that water is replaced by organic solvents for absorption of CO_2 and H_2S. Mostly products are involved in this technique named Selexol® and Genosorb®. These contain mixtures of dimethyl ethers of polyethylene glycol and methanol. The benefit of using these organic solvents rather than water is that high solubility of CO_2 could be achieved. Also, specifically, with Selexol® process, energy consumption can be lesser, e.g., approximately 15% than water scrubbing (Sun et al. 2015). This technique requires smaller upgradation units because it has the ability to absorb three times more CO_2 than water, which directly indicates that a lesser amount of liquid inputs is needed in the system. Yet, the demerit of the technique is that the organic solvents are tough to regenerate as a result of the more solubility of CO_2. In the case of H_2S separation, Selexol® is preferred because the solubility of H_2S is comparatively higher than CO_2. Due to this reason, during the solvent regeneration process, it needs high temperature. It suggests that if H_2S concentration is higher in raw biogas then it needs a higher temperature for separation. So, to save energy, pretreatment of raw gas (removal of H_2S) is suggested prior to its injection into the solvent system (Persson 2003; Awe et al. 2017).

Bauer et al. (2013a) described the system of organic scrubbing which explained in Fig. 17.4. It contains compressed raw biogas under 7–8 bars and needs to be chilled to 20 °C, prior to injection from the bottom absorption column. The organic solvent is also required to be cooled down before its inoculation with gas, as high temperature can adversely affect the Henry's constant. Bauer et al. (2013a, b) and Sun et al. (2015) suggested that regeneration of the organic solvent by heating it near about 70–80 °C and injecting the mixture in the desorption column resulted in the release of pressure up to 1 bar. This technology can be founded as a 98% methane upgrading technique (Bauer et al. 2013a, b; Sun et al. 2015).

17.2.1.3 Pressure Swing Adsorption (PSA)

Pressure swing adsorption (PSA) method contains four vertical columns packed with absorbents; the elucidation of the method is shown in Fig. 17.5. It contains sequential setup consisting of adsorption, depressurization, desorption, and pressurization as well as the molecular sieves material. When a methane-rich gas passed through the pressurized column, the CO_2 gets adsorbed by absorbents such as zeolite, activated charcoal, activated carbon, synthetic resins, and silica gel. This absorbent led to the separation of CO_2, N_2, O_2, and H_2S from the biogas. Selective absorption of CO_2 over CH_4 can be possible by feeding gas into a broad specific area containing porous adsorbents material. The CO_2 can easily absorb on the surface of porous absorbent because a molecule of CH_4 is larger than the molecules of other gas. This method is preferred for gas cooling and H_2S removal before CO_2 adsorption because the molecular sieves are capable of entrapping H_2S irreversibly (Awe et al. 2017).

Once absorption reaches a saturation point with absorbed CO_2, gas flow is directed to the depressurized column where pressure is almost at atmospheric level with saturated CO_2 column. In this column high methane containing CO_2/CH_4 mixture is vacuumed and recycled. It will be then led back to the inlet of PSA.

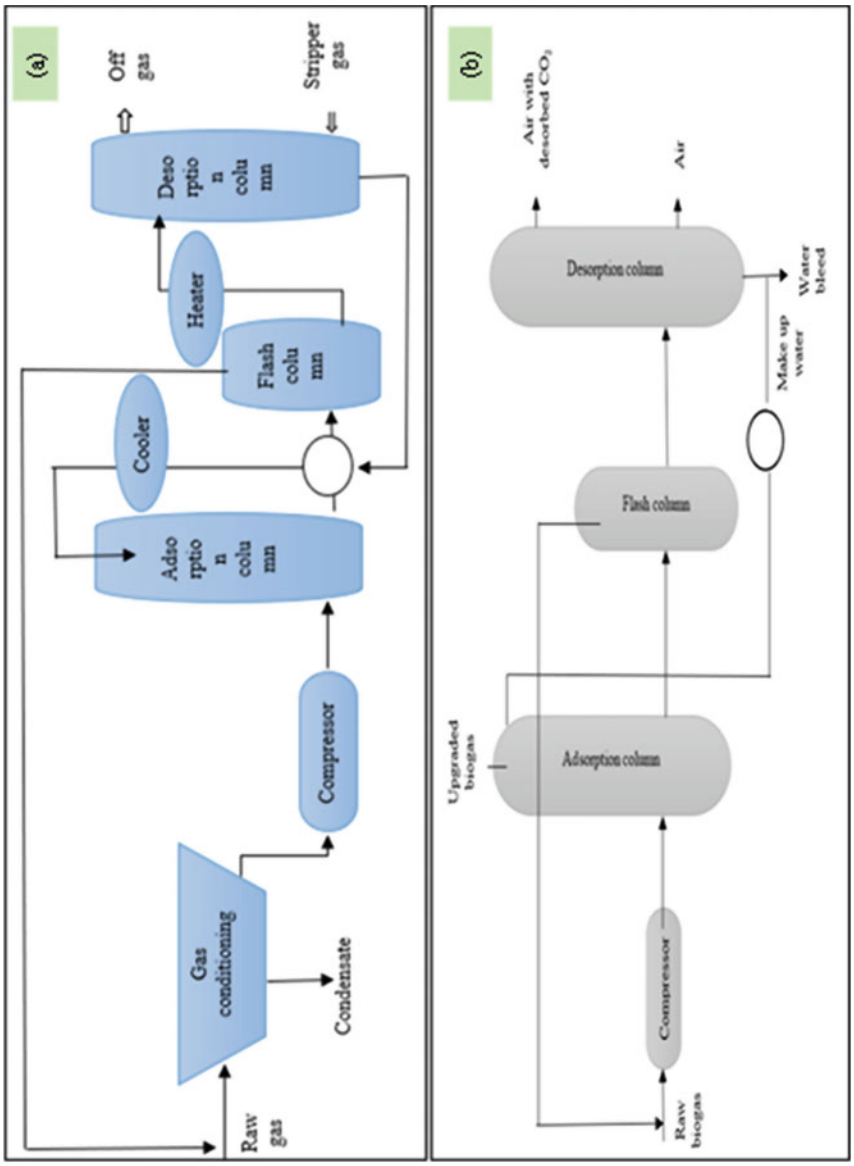

Fig. 17.4 Physical absorption via (**a**) organic and (**b**) water solvents

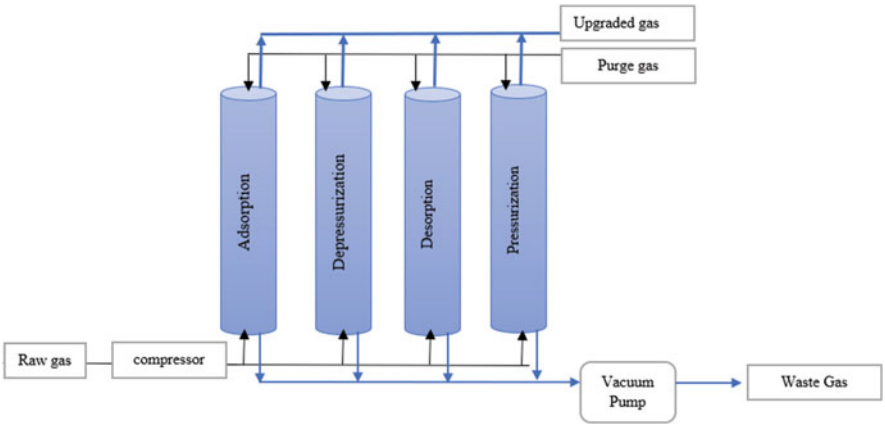

Fig. 17.5 Pressure swing adsorption

This technique reported total CH_4 upgradation in 96–98% range, with very little methane loss, i.e., 2–4% (Thrän et al. 2017). At last, the off-gas must be burnt in a flox burner to prevent the release of methane in the atmosphere, or utilization for ignition (Ryckebosch et al. 2011).

17.2.1.4 Membrane Separation

Membrane separation is the conventional, easy to process, cheap, advantageous molecular-based technology for biogas upgradation (Vilardi et al. 2020). This technique focuses on the principle of keeping the size of pores fixed according to size for CO_2 and H_2S so that CH_4 cannot pass through the membrane. Yet, some CH_4 molecules can cross the membrane so to prevent methane loss, the accuracy and purity of the membrane are mandatory. Schematic presentation of membrane separation is mentioned in Fig. 17.6. Optimization and enrichment of the membrane separation technique were attempted by Basu et al. (2010) and Scholz et al. (2013) for commercial application. Maximum suitability was exhibited by polyimide and cellulose acetate-based membranes for biogas upgradation. Selective for CO_2 polyvinylamine/poly-vinyl alcohol blend membrane was applied and tested. Under optimum condition, this blended membrane can result in 98% CH_4 purity with 99% recovery (Deng and Hägg 2010).

17.2.1.5 Cryogenic Separation Process

The temperature regulation strategy can be utilized for separation of gases. Every gas had a different boiling point, for example CO_2 and CH_4 have a boiling point of −78 °C and −160 °C, respectively (Baena-Moreno et al. 2019; Kapoor et al. 2019). Raw biogas can be separated by cooling the mixtures at desired pressure (the process flow shown in Fig. 17.7). Under known boiling temperatures gases like N_2, O_2, and siloxanes can be removed from biogas with the help of condensation and distillation. Hosseini and Wahid (2014) reported that the specific value of the final products like

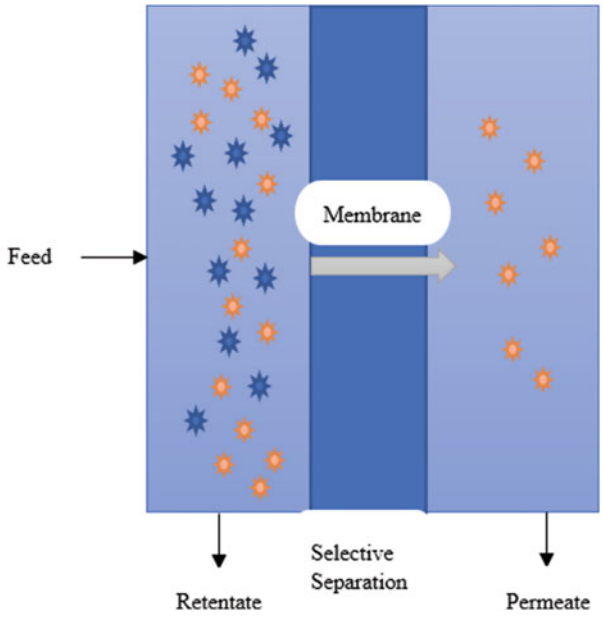

Fig. 17.6 Membrane separation

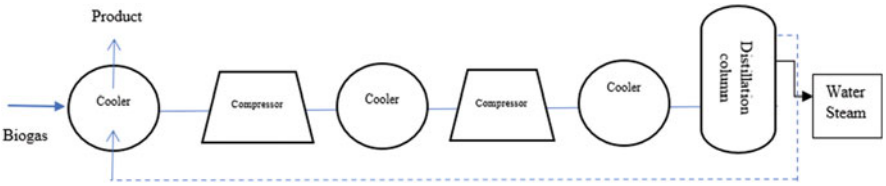

Fig. 17.7 Cryogenic separation

liquid biomethane (LBM) is equivalent to liquid natural gas (LNG) (Khan et al. 2017). This method can help in the recovery of methane in high purity with a loss of less than 1% methane, which is applicable for landfill gas treatment. Also, for CO_2 production around 98% can be utilized straight for transportation fuel as well as grinding. Cryogenic separation is energy intensive because raw biogas needs to be compressed at 200 bar, which can be resulted in production of an amount of biogas to 5–10% (Johnston 2014; Miltner et al. 2017).

17.2.1.6 Chemical Hydrogenation

Sabatier reaction is principally actioned in chemical hydrogenation process which can be also applied for biological methods of biogas upgradation. The advantage of this technique is that it is highly selective and thus can be useful in entrapping CO_2 and H_2 (Jürgensen et al. 2014). Many catalysts such as nickel and ruthenium were reported for this technique which is advantageous in terms of industrial aspects (Jürgensen et al. 2015). Xia et al. (2016) suggested that high temperature around

300 °C and 5–20 MPa pressure levels were optimal for biogas upgradation. However, the disadvantages of this method are low success of catalyst (Guebitz et al. 2015), high cost of catalysts as well as energy intensive nature that limits the sustainability of the functioning of the process (Fu et al. 2020).

17.2.2 Biological Technologies

When the anaerobic microorganisms are involved in anaerobic digestion of different types of substrates, it resulted as raw biogas. The raw biogas can later be subjected to upgradation to eliminate constituent impurities. There are two major groups that were reported as well as still in practice for biogas upgradation, e.g., photoautotrophic and chemoautotrophic (Wu et al. 2017). Both groups can be dependent on surroundings and useful for biogas upgradation. Details of merits and demerits are mentioned in Table 17.2.

Hydrogenotrophic methanogens were mostly reported for uptake of CO_2 and utilize H_2 for the translation of CO_2 to CH_4 based on the reaction as shown in Eq. (17.1).

Table 17.2 Biological methods (Fu et al. 2020)

Organisms	Mechanism	Merits	Demerits
Photoautotrophs	Photosynthesis CO_2 + solar light exposure + water + nutrient → biomass + oxygen + heat	✓ CO_2 can convert into valuable products and recover high methane near about 97% ✓ Water and land required in low quantity ✓ Active biomass yielded	✗ Very expensive and high energy requirements ✗ Due to dependency of natural processes photosynthetic rate can slow down and uptake of CO_2 delayed ✗ Microbial contamination.
Chemoautotrophs	Methanogenesis process CO_2 consumption taken place by methanogens	✓ Selectivity, efficient process, and pure CH_4 recovery ✓ CO_2 consume and convert into CH_4 ✓ Atmospheric pressure and temperatures mostly suitable ✓ Easy adaptation in anaerobic processes ✓ Approachable method for environment.	✗ Large volume of reductant required ✗ This method is still under development

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad \Delta G^{\circ} = -130.7\,\text{kJ/mol} \tag{17.1}$$

The solar and wind energy mediated electricity generation can be used for the hydrolysis process since water hydrolysis is mandatory because of the H_2 requirement. However, H_2 can be tough to store due to its low energy density (Jürgensen et al. 2014). This H_2-directed biogas upgrading method can be divided into biological in situ and ex situ biogas upgrading. Ex situ upgrading needs CO_2 to be removed first whereas the in situ method does not need CO_2 removal at the beginning (Singhal et al. 2017; Kougias et al. 2017). Various bioreactors were put in practice since the development of this method, depending on hydrogenotrophic methanogenesis and Wood–Ljungdahl pathways (Kougias et al. 2017; Stams and Plugge 2009). Hydrogenotrophic methanogenesis helps in the generation of CH_4 from CO_2, with the H_2 as a source of electrons, according to Eq. (17.1). The Wood–Ljungdahl pathway follows two-step reactions, as written here into Equations (17.2) and (17.3) (Adnan et al. 2019).

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad CH_3COOH \rightarrow CH_4 + CO_2 \tag{17.2}$$

$$\Delta G^{\circ} = -104.5\,\text{kJ/mol} \quad \Delta G^{\circ} = -31.0\,\text{kJ/mol} \tag{17.3}$$

Control of H_2 concentration during the process is necessary due to the equilibrium of metabolic biochemical reactions. The equations clearly show that CO_2 is converted to acetate via homoacetogenic bacteria. Later, acetate is converted into CH_4 in the presence of acetoclastic methanogenic archaea (Schuchmann and Müller 2014).

Upgradation reactors reported for in situ methods are batch reactors, CSTR, and continuous UASB and for ex situ membrane bioreactor, CSTR up-flow reactors, bubble column reactor with batch and continuous, UASB continuous, and trickled bed reactor (Kapoor et al. 2019).

17.2.2.1 Microorganisms Involve in Biogas upgrading Systems

Classification of microbes for this category depends on the utilization of substrate. Methanogens can be generally classified into three groups: hydrogenotrophic, aceticlastic, and methylotrophic. Methanogenesis process is dependent on various parameters, i.e., pH and temperature. Likewise, substrate and anaerobes can also be considered as prime factors (Ganesh 2014).

Hydrogenotrophic methanogens uptake substrates such as H_2, CO_2, formate, and methanol. The reaction performed is shown in Eqs. (17.4), (17.5), (17.6).

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \tag{17.4}$$

$$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O \tag{17.5}$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \tag{17.6}$$

Aceticlastic methanogens could utilize acetate as a major substrate. The reaction performed is shown in Eq. 17.7.

$$CH_3COOH \rightarrow CH_4 + CO_2 \tag{17.7}$$

Methylotrophic methanogens utilize substrates like trimethylamine, dimethyl sulfate, and methylated ethanolamines. Equations (17.8), (17.9), (17.10), and (17.11) show the reaction carried out there.

$$4(CH_3)_3N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3 \tag{17.8}$$

$$2(CH_3)_2NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3 \tag{17.9}$$

$$4(CH_3)NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3 \tag{17.10}$$

$$2(CH_3)_2S + 2H_2O \rightarrow 3CH_4 + CO_2 + H_2S \tag{17.11}$$

The most reported hydrogenotrophic methanogens are *Methanobacterium bryantii, Methanobacterium formicicum, Methanobacterium thermoalcaliphium, Methanothermobacter thermoautotrophicum, Methanothermobacter wolfeii, Methanobrevibacter smithii, Methanobrevibacter ruminantium,* and *Methanococcoides methylutens* from deep marine sediments, termite hindguts, human gastrointestinal tracts, and animal gastrointestinal tracts.

Aceticlastic methanogens include *Methanosaeta concilii (soehngenii)* and *Methanosaeta thermophila* mostly isolated from habitats like anaerobic digesters, rice fields, and wetlands.

Methylotrophic methanogens are *Methanosarcina barkeri, Methanosarcina mazei, and Methanosarcina thermophile* that could be identified from marine, hypersaline, sulfate-rich sedimental areas (Agneessens et al. 2017; Bassani et al. 2017; Luo and Angelidaki 2013; Mulat et al. 2017; Adnan et al. 2019).

17.2.2.2 Biogas upgrading Through Other Fermentation Processes

The fermentation-assisted upgradation of CH_4 is an advantageous method because of its ability to generate several value-added products as by-product. These value-added products such as ethanol and butanol can be used as biofuel. Also commercially important fatty acids like acetate and butyrate are also produced during the process (Agler et al. 2011; Kennes et al. 2016; Martin et al. 2016; Zhang et al. 2013). Several microorganisms such as *Acetobacterium woodii, Butyribacterium methylotrophicumn,* and *Clostridium scatologenes* have demonstrated biogas upgradation potential (Schiel-Bengelsdorf and Dürre 2012) channeled by Wood–Ljungdahl pathway (Latif et al. 2014). Trickle bed reactor with *Clostridium ragsdalei* is utilized for ethanol production from syngas amended with H_2 and CO_2, as the microbial catalyst. The limitation of fermentation technique is low H_2 solubility and higher energy requirement to gain high gas-liquid mass transfer (Luo et al. 2012; Devarapalli et al. 2016).

17.2.2.3 Biogas Upgrading Through Microbial Electrochemical Methods

Bioelectrochemical (BEC) methods are trending nowadays due to their ability to remove CO_2 from biogas to have high yield of CH_4. BEC methods have evolved as a sustainable and profitable technique to upgrade biogas (Lovley and Nevin 2013; Van Eerten-Jansen et al. 2012). In a microbial electrolysis cell (MEC), H_2 is formed on cathode and later used for biogas upgradation. Cheng et al. (2009) suggested that the reduction of CO_2 on cathode via biocathode in MEC reactor and achieved 80% CH_4 recovery. In situ cathode containing MEC was used as a biogas reactor and ex situ containing MEC was demonstrated for CO_2 removal. The experiment result suggested that the in situ technique was better than the ex situ technique (Xu et al. 2014). Lately, by Zeppilli et al. (2016), another technique was demonstrated, in which a separate chamber was used for the CO_2 removal. The anion exchange membrane (AEM) and proton exchange membrane (PEM) equipped MEC were compared for the removal of CO_2 from simulated biogas.

17.3 The Feasibility of Biogas Upgrading

In biomethanation reaction, H_2 electrolysis and methanation are required to figure out the costs of functioning and investment involved. Small-scale experimental designs can be upscaled to large-scale plants for biogas upgradation to achieve pure CH_4 gas for commercial application. Cost for the construction and process can be estimated by evaluation of the design, materials, and methods. The gap between small-scale and large-scale production needs to be feasibly resolved to get maximum production. The aforementioned technologies have the potential to upgrade biogas with optimized and strategic operation.

17.4 Future Aspects

The application of upgraded biogas from raw biogas is needed in huge amount in various fields. Several methods are still under development to be commercial applied for upgrading biogas. Biogas can be upgraded to bio-CNG and other fuels, energy, and value-added products via upgradation. Conversion via in situ CO_2 to CH_4 signifies a capable area for biogas upgrading due to its qualities. For upgradation of biogas quality in terms of CH_4 percentage and heating potential, several easy, sustainable, economical, and environmentally viable physicochemical and biological methods need to be developed that can easily entrap and utilize carbon dioxide and other constituent impurities from biogas.

17.5 Conclusion

The present chapter concludes that upgradation technologies can help to improve utilization and conversion of methane. As pure CH_4–containing biogas is more reliable for utilization at the large scale application level, biogas must be upgraded for utilization. The developed or developing methods are able to upgrade biogas at their own efficacy with associated benefits and demerits. Physicochemical methods are easy to operate but are not cost effective, and biological methods are slower but given the high recovery of methane it is considered environmentally sustainable. Microbes involved in the process of microbe-assisted upgradation can utilize impurities of biogas like CO_2, H_2S, and N_2 and convert or generate CH_4 via hydrolysis, methanogenesis, and fermentation pathways. This study suggests that further research needs to be done for overcoming the limitations of existing technologies.

Declaration of Conflict of Interest There is no conflict of interest to declare.

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Microbial Bioelectricity Generation and Product Electrosynthesis

18

John M. Pisciotta and Samantha Blessing

Abstract

Electrically active microorganisms provide diverse methods for converting available energy, like sunlight and organic waste, into more valuable forms. This chapter examines the role of electrically active microorganisms in treating waste while generating bioelectricity or storing renewable energy as useful chemicals. Wastewater treatment is currently an energy-intensive process that can be conducted in concert with microbial electricity generation. Organic waste can be efficiently converted to direct current (DC) electricity using microbial fuel cells (MFCs). We review the different types and configurations of MFCs including heterotrophic and photosynthetic MFCs. Achievable power densities generated by disparate electrogenic microorganisms on the bioanode are examined. Newer bioelectrochemical systems (BESs) harness electrotrophic microbes at biocathodes for storing energy as green hydrogen or electrosynthesis of organic products from CO_2. Anaerobic digestion of waste produces biogas methane, a process that can be enhanced using electromethanogenesis.

Keywords

Biocathode · Bioelectricity · MFC · Hydrogen · Electrosynthesis · Electrofermentation

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_18

Abbreviations

ACS	Acetyl-coenzyme A synthase
AEM	Anion exchange membrane
APB	Anoxygenic photosynthetic bacteria
BES	Bioelectrochemical system
CB	Calvin-Benson
CEM	Cation exchange membrane
Chl	Chlorophyll
CNT	Carbon nanotube
COD	Chemical oxygen demand
CODH	Carbon monoxide (CO) dehydrogenase
DC	Direct current
ICE	Internal combustion engine
LH	Light harvesting
MDC	Microbial desalination cells
MEC	Microbial electrolysis cell
MFC	Microbial fuel cell
MHC	Multi-heme cytochrome
mW/m^2	milliwatts per square meter
NPQ	Nonphotochemical quenching
PEM	Proton exchange membrane
PETC	Photosynthetic electron transport chain
photoMFC	Photosynthetic microbial fuel cell
plant-MFC	Plant microbial fuel cell
SHE	Standard hydrogen electrode
SLAP-MFCs	Soil-less aquatic plant microbial fuel cells
SRB	Sulfate-reducing bacteria
TAG	Triacylglycerol
VFA	Volatile fatty acid

18.1 Introduction

Bioelectrochemical systems (BESs) are composite devices that use microorganisms and/or enzymes to generate or consume electric current for a range of applications from power production to bioremediation to biofuel formation. Purely enzymatic BESs cannot undergo self-repair, unlike microbial BESs which use living microorganisms and therefore are of more restricted industrial utility. BESs generally involve an electrolyte-immersed anode and cathode connected to one another by way of an external circuit. In some embodiments, these electrodes may be separated from one another by one or more ion exchange membranes. BESs are finding an ever-increasing number of niche applications throughout society. These range from

the real-time detection of blood glucose levels in diabetics to the efficient desalination of seawater. Two types of BESs that are gaining interest in the industry for renewable energy applications are microbial fuel cells (MFCs) and microbial electrolysis cells (MECs). MFCs use electrogenic microbes to generate renewable electricity from the microbial catabolism of organic material in waste. MECs and electrosynthetic reactors use electrotrophic microbes to produce a diversity of industrially useful molecules. Here we review the design and operation of different BESs, the microbes involved, and opportunities for future industrial use of BESs throughout society. Particular focus is on waste treatment and renewable energy storage.

18.2 Microbial Electricity Generation

Microbial generation of electricity has been investigated for over a century, while the development of MFCs for energy production has been studied for about 50 years (Cao et al. 2019). Potter (1911) was the first to explore the feasibility of using microorganisms in electrical generation. Potter used a fluid-filled jar for housing one platinum electrode and a porous cylinder for the other, forming a biological galvanic cell. He found that adding a culture of *Saccharomyces* yeast or bacteria under the appropriate conditions set up an electrochemical potential. Early work on bioelectrochemical systems (BESs) gave rise to microbial fuel cells (MFCs) (Ng et al. 2014). MFCs are BESs that use microbes as biocatalysts to generate electrical current (Rosenbaum et al. 2010). Biocatalysts are less expensive than the precious metal electrode catalysts used for traditional fuel cells (FCs) and therefore represent an appealing means of electricity generation (Mokhtarian et al. 2012). The organic fuel used is biodegradable and is oxidized by microorganisms in the anode chamber to provide electrons to the circuit (Bond and Lovley 2005). As MFCs have developed over the past few decades, their potential for generating higher current densities has increased (Allen and Bennetto 1993). Newer types of MFCs that do not depend on organic molecules have been developed. For example, photosynthetic microbial fuel cells (photoMFCs), sometimes called bio-photovoltaics (BPVs), employ light energy captured by phototrophs to produce electricity (Mao and Verwoerd 2013). MFCs have been used in sustainable electricity generation and wastewater treatment. They function under mild conditions and use a large variety of versatile biological materials as fuel. Most MFCs use inexpensive electrode materials, such as graphite, and bacteria as biocatalysts at the anode surface to promote a cost-effective way of generating electricity (Cao et al. 2019; Chaturvedi and Verma 2014, 2016).

18.2.1 Microbial Fuel Cells

MFCs are superficially similar to conventional batteries in that both use conductive electrolytes, an anode, and a cathode that in many cases are separated by a proton

exchange membrane (PEM) (Cao et al. 2019; Ghasemi et al. 2013; Ucar et al. 2017). However, in a conventional battery, the anode is abiotic, whereas in MFCs a bioanode covered with electrogenic microbes is used to develop the electrical potential. The bioanode is where electrogenic, anaerobic microorganisms deposit electrons as they oxidize organics, releasing protons plus CO_2. Electrons flow from microbe to anode creating a voltage and direct electrical current that flows to the cathode via an external electrical circuit (Ucar et al. 2017; Agrawal et al. 2019; Kumar et al. 2018). Protons diffuse through the PEM to the cathode chamber, and, at the cathode, an external inorganic electron acceptor, oxygen in aerobic MFCs, reacts with electrons and protons to form the inert end product H_2O as shown in equation:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \tag{18.1}$$

Oxygen in the anode chamber inhibits heterotrophic electricity production by anaerobic electrogens, requiring the prevention of oxygen entering the anode chamber (Najafpour et al. 2011). To separate the anode and cathode chambers, a membrane (i.e., PEM) is often positioned between the chambers. Membranes allow for proton exchange between the anode and cathode chambers while inhibiting substrate and oxygen permeation between chambers (Nandy and Kundu 2018). Membrane materials popularly used in MFCs include Nafion and Ultrex (Nandy and Kundu 2018); Chaturvedi and Verma 2014). Selective anion exchange membranes (AEM) or cation exchange membranes (CEM) are occasionally used but tend to drive up costs. Membrane-less MFCs avert such costs but may be prone to oxygen inhibition of anaerobic electrogenic bacteria at the anode. A traditional-style two-chamber MFC featuring a PEM is shown in Fig. 18.1.

Key parameters monitored to evaluate MFC performance include cell voltage (V), current density (mA/m^2), internal resistance (Ohms), and power density (mW/m^2) (Rahimnejad et al. 2015). Reactor configurations influence the microbial populations enriched in BESs and may bolster MFC performance. Anode-enriched microorganisms are typically electrogenic and function as biocatalysts in the anode chamber of MFCs. The anaerobic anode chamber contains the requirements for biomass degradation including substrate, mediator (if applicable), the microorganism(s), and the electron acceptor in the form of the anode electrode (Rahimnejad et al. 2015). Electrons produced are passed from electrogens to anode and through an external electrical circuit to the cathode (Ucar et al. 2017). Microorganisms act as catalysts for the reactions because the activation energy must be lowered for the anodic reactions to take place (Hassan et al. 2014; Virdis et al. 2011). The MFC anode material should be non-fouling, noncorrosive, and conductive and contain a high surface area to encourage microbial adhesion and growth (Logan 2008; Nandy and Kundu 2018). Anode materials used are generally carbon-based including carbon paper, carbon cloth, carbon felt, reticulated vitreous carbon (RVC), graphite rod, or graphite fiber brush (Rahimnejad et al. 2015). Useful electrode materials have high electrical conductivity, large surface areas, and low cost and are sustainable (Logan et al. 2006; Wei et al. 2011). Modified carbon or polymers have also been considered a successful alternative to carbon or metallic electrodes (Niessen et al.

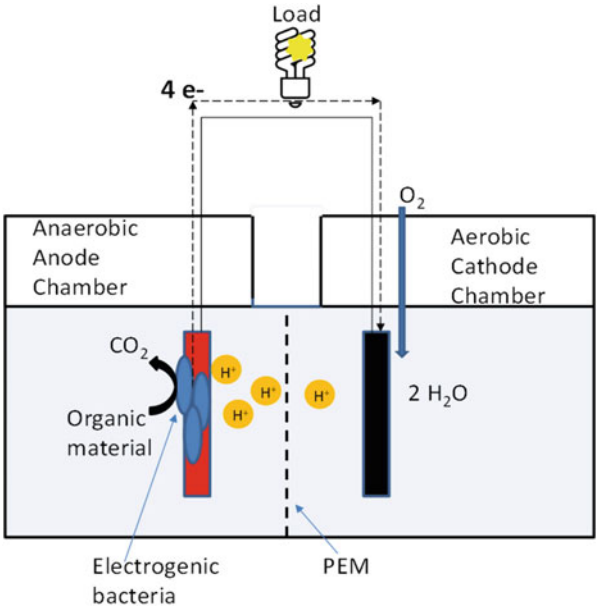

Fig. 18.1 Dual-chamber MFC featuring a PEM separating anaerobic anode chamber from aerobic cathode chamber. Electrogenic microbes on the anode catabolize organics, generating CO_2 and protons as electrons are donated to the anode, building up a voltage that flows as DC to the cathode. Electrons exit the cathode and reduce oxygen in the presence of the arriving protons for end product water

2004). In certain types of MFCs, electron transfer allows for the removal of organic waste or pollutants, as occurs in various sediment microbial fuel cells (sMFCs) (Pisciotta and Dolceamore Jr 2016). Researchers from the lab prepare to install scaled-up sMFCs in a Pennsylvania pond in Fig. 18.2 to study autochthonous electrogens under natural conditions. Many studies have examined MFCs under laboratory conditions, and far fewer have investigated MFC performance under real-world conditions. That will be needed to industrialize BESs generally.

Electric current generation is dependent on the microbially mediated substrate oxidation rate and the efficiency of electron transfer to the electrodes (Mokhtarian et al. 2012). Mechanisms electrogens use to transfer electrons to anode include (1) indirect electron transfer using electron mediators and (2) direct electron transfer through contact between the electrode and cell surface (mediator-less) (Cao et al. 2019). Niu et al. (2020) found that *Klebsiella oxytoca* d7 could attach to the anode and secrete electron transport mediators (ETM) which enabled a power density of 4912 mW/m^2. When the direct anode contact of *K. oxytoca* d7 was removed and a barrier was placed between the microbe and the anode, a power density of 1481 mW/m^2 was obtained using solely the ETM for electron transport (Niu et al. 2020). Mediators are commonly used for model electrogens such as *Shewanella* spp., as well as for *Pseudomonas* spp., *Escherichia coli*, and *Bacillus* spp. (Mokhtarian et al. 2012). Unfortunately, common soluble mediators, such as ferricyanide, are toxic to many organisms and unsuitable for water treatment so improved methods are needed. One approach is to optimize the electrogenic anode community and the use of mediator-less MFCs for heightened catabolic rates and improved power output. Examples of power densities generated by various species of microorganisms tested in MFCs between 2004 and 2021 are shown in Table 18.1.

Fig. 18.2 MFC field researchers preparing to install large sediment MFCs (sMFCs) in a pond to enrich naturally occurring electrogens from sediment on the lower graphite anodes and phototrophic, biocathode-associated microbes on the upper carbon cloth cathodes. Left to right: John Pisciotta, Paige Minka, and Jeremy Irving, photo by Nikita Panov

Electrogenic bacteria that perform direct electron transport include *Shewanella putrefacien*s and *Aeromonas hydrophila* (Mokhtarian et al. 2012). With direct electron transfer to the anode, electrochemically active species form a biofilm typically involving nanowires for anode attachment, and electron transfer occurs via outer cytochromes (cyt) and nanowires or by using direct contact with transmembrane electron transport proteins (Reguera et al. 2005). Electron transport proteins are responsible for transferring electrons from the cytoplasm to the outer cell membrane and finally the anode (Cao et al. 2019). Some electrogenic bacteria have external membrane pili that assist in electron transport (Mokhtarian et al. 2012).

Table 18.1 Table showing reported power densities using various MFC designs from 2005 to 2021

Organism	Chamber	Anolyte/ substrate	Anode	(mW/m^2)	References
Wastewater	Single	Wastewater	Pierced CP	1330	Liu et al. (2005)
Ochrobactrum anthropi YZ-1	Dual	Acetate	CC	89	Zuo et al. (2008)
Acidiphilium cryptum	Dual	Glucose	Graphite felt	12.7	Borole et al. (2008)
Geobacter sulfurreducens	Dual	Acetate	CC	1880	Nevin et al. (2008)
Escherichia coli	Dual	Glucose	CC	1304	Xiang et al. (2009)
Aerobic and anaerobic sludge	Dual	Acetate	CC/bamboo-N-doped CNT	1040	Ci et al. (2012)
Rhodospirillum rubrum	Dual	Nutrient broth, LB with glucose	CC	1250	Gomez et al. (2014)
Escherichia coli	Dual	LB medium	CP/Fe_3O_4-CNT	830	Park et al. (2014)
Escherichia coli	Dual	Glucose	MWCNTs-SnO_2/ GCE	1421	Mehdinia et al. (2014b)
Escherichia coli	Dual	Glucose	CC/GO-SnO_2	1624	Mehdinia et al. (2014a)
Shewanella putrefaciens	Dual	Lysogeny broth	CC/NiO	1024	Qiao et al. (2014)
Escherichia coli	Dual	Glucose	CC+ PANI/rGO/ Pt	2059	Gnana Kumar et al. (2014b)
Escherichia coli ATCC 27325	Dual	Glucose	CC + polypyrrole/ rGO	1068	Gnana Kumar et al. (2014a)
Shewanella oneidensis MR-1	Dual	Tryptic soy broth	SSM/CNT	450	Erbay et al. (2015)
Mixed culture	Dual	Acetate	SSM/CB	3215	Zheng et al. (2015)
Shewanella putrefaciens	Dual	LB medium	CC/TiO_2/rGO	540	Zou et al. (2015)

(continued)

Table 18.1 (continued)

Organism	Chamber	Anolyte/ substrate	Anode	(mW/m^2)	References
Geobacter-enriched mix	Dual	Acetate	3D macroporous graphene	5600	Ren et al. (2016)
Geobacter-enriched mix	Dual	Acetate	2D single-layer graphene	2460	Ren et al. (2016)
Shewanella putrefaciens CN32	Dual	PANI	LMC	1280	Zou et al. (2017)
Shewanella putrefaciens CN32	Dual	LB medium	CC-NiO/graphene nanocomp.	3632	Wu et al. (2018)
Geobacter sp.	Dual	Acetate	FeS_2/rGO nanop graphene	3220	Wang et al. (2018b)
Exoelectrogen mixed culture from wastewater	Single	Acetate	CF	2000	Jiang et al. (2020)
Soil mixed community	Single	Urine	CF	126	Simeon et al. (2020)
Mix, *Geobacter* and *Holophaga* sp.	Dual	Acetate	$MnFe_2O_4$/CF	3836	Xue et al. (2021)

Other mediator-less MFCs can run on wastewater, such as sewage containing wastewater as this process is accomplished without the use of any diffusional electron mediators (Parkash 2016). Electrode treatment with certain acids, such as phenylboronic acid, may promote anode biofilm adhesion and development (Lapinsonnière et al. 2013). The overall performance of a direct transfer MFC is reliant on the transfer of electrons between the bacterial cell membrane and the electrode surface. Species capable of direct electron transfer to the MFC anode include *Geobacter sulfurreducens*, *Shewanella putrefaciens*, and *Rhodoferax ferrireducens* (Bond and Lovley 2003; Kaur et al. 2020; Park and Zeikus 2002). *Ochrobactrum also possesses electrogenic activity* (Zuo et al. 2008). *Certain archaea have limited electrogenic activity* (Abrevaya et al. 2011). *G. sulfurreducens* is the most extensively studied model electrogen. This is because of *G. sulfurreducen*'s natural ubiquity and high current output attributable to multi-cell layer thick electrode biofilm containing conductive cytochromes and microbial nanowires (Bond and Lovley 2003; Reguera et al. 2005).

Higher DC is possible with systems that use soluble chemical mediators as electron shuttles (Allen and Bennetto 1993; Park and Zeikus 2000; Rabaey et al. 2005). Mediator usage is necessary for microorganisms that do not contain electrochemically active surface proteins or other endogenously produced factors to aid in electron transfer between microbe and anode (Flimban et al. 2019). Widely used synthetic mediators include methyl blue, neutral red, and thionine (Kaur et al. 2020; Park and Zeikus 2000). Soluble mediators may be recruited to achieve electron

transfer without direct cell to electrode contact (Cao et al. 2019). This can occur by electron mediators entering the cell and harnessing electrons from metabolic reactions, delivering them to the anode (He et al. 2017). Biological mediators are endogenously produced by certain microorganisms and include phenazine and pyocyanin (Bilal et al. 2018; Cao et al. 2019; Debabov 2008; Peng et al. 2018; Rabaey et al. 2005). Electron mediators produced by microbes such as *Pseudomonas*, *Enterococcus*, and *Lactobacillus* promote redox electron shuttling to the MFC anode (Pandit et al. 2017). Some electron mediators or electron acceptors that are used include ferricyanide, anthracenedione, persulfate, and manganese dioxide, among others (Aelterman et al. 2006; Kaur et al. 2020; Li et al. 2009; Rabaey et al. 2003; Rhoads et al. 2005; Schröder et al. 2003; Ucar et al. 2017). Although mediators can improve power output, many are a cause of concern due to their toxic effects and environmental risks which must be considered when developing MFC technology for large-scale applications.

Power generation depends on additional factors besides the microbe(s) utilized. Oxygen supply and consumption rates in the cathode chamber, substrate oxidation rate in the anode chamber, and internal resistance to proton transfer related to PEM permeability all influence and may limit power production (Sharma and Li 2010). Materials used, MFC fuel substrate and loading rate, electrode material, mediator used (if applicable), pH, temperature, ionic strength of the system, and electrode and circuit configuration can influence effectiveness performance (Logan et al. 2006; Mokhtarian et al. 2012; Oh et al. 2004). Optimization of all factors is necessary for peak, sustained power generation in an MFC (Kim et al. 2007). Anodic electron transfer remains a key factor in MFC performance, and applying additional parameters such as the use of mediators, specific substrates, and media for more efficient electron transfer rates is useful but can drive up cost (Huggins et al. 2014). Employing materials with high electrical conductivity, low resistance, favorable compatibility to microorganisms, chemical stability, larger surface areas, and sufficient material strength is important (Logan et al. 2006). Materials selected for an MFC system should maximize current density, power density, and coulombic efficiency while being cost-effective and corrosion-resistant (Gadkari et al. 2019; Iannucci et al. 2018). MFCs should have a lifetime of up to 5 years or greater (Kim et al. 2003; Moon et al. 2006).

Overpotentials and charge transfer resistances between the bacteria and electrode can hinder proton and electron transport and affect the overall efficiency of the MFC system (ElMekawy et al. 2013). Internal and external resistance to charge transfer of electrons and protons are important considerations when designing an MFC system. A system's electrical resistance relates to the hindrance of the flowing electrical current through the system (Kamau et al. 2017). In an electrical system such as an MFC, factors affecting resistance include surface area, distance, and electrical resistivity (Ω). When analyzing the internal resistance of an MFC, the measured voltage output of a cell is lower with a current than when no current has been supplied because of the flow of electrons (Kamau et al. 2017). Proton diffusion to the cathode can also be inhibited as a form of internal charge transfer resistance. Protons must balance electrons at the cathode for sustained oxygen reduction. An MFC can

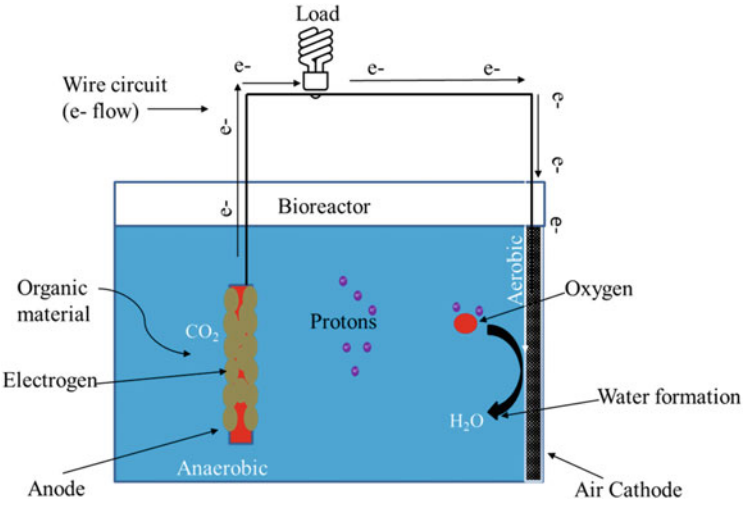

Fig. 18.3 Air cathode single-chamber MFC. Electrogenic microbes on the anode catabolize organics, generating CO_2 and protons as electrons are donated to the anode, building up a voltage that flows as DC to the cathode. Electrons exit the cathode and reduce oxygen in the presence of the arriving protons for end product water

face internal resistance at the anode or cathode or from the electrolyte (or membrane) (ElMekawy et al. 2013). Menicucci et al. (2006) found that when external resistance decreases, cell voltage also decreases which can be attributed to limitations through reaction kinetics at an electrode, mass transfer, and charge transfer activities occurring at the current-limiting electrode. Cell voltage increase was observed when internal resistance was increased through a study by Ghangrekar and Shinde (2007) and similar results by Rismani-Yazdi et al. (2011). The use of different external resistors can alter anode potential, and higher anode potentials were observed in MFCs with lower external resistances (Kamau et al. 2017). Cell potential acclimates with the external resistance, and when considering the maximum power transfer theorem, the MFC power density is highly dependent on the electrical current in the system (Kamau et al. 2017). Essentially, a difference in external resistance and internal resistance causes a typical decrease in power generation. This theorem is not universal. Lee et al. (2010) observed larger power densities with increased external resistance. Overall resistance is dependent on a number of factors related to the whole system including biofilm structure and species, current density, coulombic efficiency, and anode potential (Kamau et al. 2017). An air cathode MFC that dispenses with the PEM is depicted in Fig. 18.3.

Metal catalysts are frequently used but also drive up costs. In a dual-chamber MFC, protons diffuse through the PEM from the anode to the cathode (Ucar et al. 2017). Protons react with oxygen as it is reduced by electrons exiting the cathode to form water, while steady electrical current streams through the external metal wire connection (Bettin 2006). The DC through the external wire can be used to do work. Metal catalyst (e.g., platinum) or enzymatic catalyst on the cathode is typically used to increase the rate of the cathode oxygen reduction reaction, although new platinum

group metal-free catalysts are also being used (Costa de Oliveira et al. 2020). The reaction rate is dependent on the oxidant, availability of protons generated from the anode, catalyst efficiency, electrode composition, and ability to catalyze the cathodic reactions (Rahimnejad et al. 2015). Catalyst reduced activation energy at both electrodes (Rahimnejad et al. 2015). In the cathode chamber of MFCs, just as in aerobic human muscle cells, oxygen is the most favorable electron acceptor because of its atmospheric availability, high redox potential (+820 mV), and its harmlessness (Rahimnejad et al. 2015; Watanabe 2008). A biocathode can be used to overcome the need for platinum-mediated oxidation of oxygen at the cathode (Fornero et al. 2008).

Organic fuels, such as acetic acid, are of low cost, are present in wastewater, and so are often used to feed MFCs. They are found naturally in combination with different electrogenic microorganisms, such as *Geobacter* spp., and mediators, like humic substances. Other organic substrates used to fuel MFCs include glucose and lactate (Prasad and Panda 2018). Glucose, though expensive, tends to be a common substrate used in the laboratory evaluation of MFCs as 24 electrons are released from the conversion of one mole of glucose to carbon dioxide (Mokhtarian et al. 2012). A wide variety of substrates can be catabolized in MFCs including hexose and pentose polysaccharides, pectin, chitin, and diverse wastewaters from food-processing, breweries, and municipal wastewaters (Chaturvedi et al. 2014; Chaturvedi and Verma 2016; Pant et al. 2010; Prasad and Panda 2018).

Applications that make MFCs an appealing technology continue to expand. Bioelectricity remains the most popular use, although power output is comparatively low (Logan 2008). MFC advancements are improving the overall power outputs and widening the potential applications and markets to which MFC technology may be applied (Gajda et al. 2018). Some of these advances relate to bioreactor size including miniaturized designs, MFC stacks combining multiple units for increased power production, and large-scale studies (Gajda et al. 2018). Because of the sustainable nature of these systems, they remain an intriguing technology for electrical power generation. MFC use in wastewater treatment has become an appealing technological application. Wastewaters in the form of food production wastewater, sanitary wastewater, and refinery wastewater among others contain quantities of usable biodegradable organic substrate (Du et al. 2007; Kumar et al. 2019). MFCs can be harnessed to remove nitrogen and organic matter at a low cost of use or for the generation of electricity from waste components through anaerobic digestion methods using sediment microbial fuel cells (sMFCs) (Gotvajn et al. 2009; Logan 2008; Mehmood et al. 2009). These have also shown success in the removal of sulfides from wastewaters along with the removal of COD by up to 90% with coulombic efficiency of up to 80% (Kim et al. 2005; Puig et al. 2011; Rabaey et al. 2006; Wang et al. 2012). Kim and Logan (2011) demonstrated desalination of synthetic seawater using microbial desalination cells (MDC), obtaining a 98% efficiency in saline removal. Powering small systems and wireless sensors that have a low power requirement capable of transferring signals such as temperature, pollutant analysis, and toxin levels are also possible (Chang et al. 2005). MFCs have the potential to be used in local areas for distributed power such as underdeveloped

areas of the world (Mokhtarian et al. 2012). Glucose sensors of different types have also been developed for use with MFCs along with the potential for petroleum and phenols toxin remediation (Bettin 2006; Luo et al. 2009; Morris and Jin 2007). This technology has also been considered for use on a spacecraft utilizing bioelectricity through wastes produced from the usage of the craft (Berk and Canfield 1964; Du et al. 2007).

The twenty-first century has witnessed diversification and improvement of MFC technologies, but challenges and limitations remain. These include slow start-up, low power output, and resistance of proton transfer by the membrane material (Chen et al. 2008). The following challenges highlighted MFC power generation issues: (1) a direct relationship between the concentration of substrate and sustained power generation and (2) usage of internal power by the bioreactor system which can limit power generation of the MFC (Kim et al. 2008; Sharma and Li 2010). Sharma and Li (2010) report the PEM can be a significant cause of internal resistance in the MFC as it slows the charge transfer of protons. To improve the power generation, single-chamber MFCs have been used, removing the PEM and reducing internal resistance caused by the membrane (Sharma and Li 2010). A system cannot reach theoretical potentials due to internal losses including ohmic, concentration, and activation losses (Kim et al. 2008). Larger surface areas of electrodes can decrease activation losses, determining appropriate distancing between electrodes can alleviate ohmic losses due to internal resistances, and monitoring concentrations of dissolved species, specifically dissolved oxygen, can lower concentration losses (Chaudhuri and Lovley 2003; Jang et al. 2004; Kim et al. 2008). Power losses also stem from MFC resistance, microbial electron transport processes, electrolyte resistance, and losses at the anode or cathode, charge transfer and membrane resistance, and electron acceptor reduction losses (Kim et al. 2008).

18.2.2 Photosynthetic Microbial Fuel Cells (PhotoMFCs)

Solar energy systems that harness biologically based energy conversion technologies are of growing significance with interest keenly focused on photosynthetic bioelectrochemical systems. PhotoMFCs use photosynthetic microorganisms added to the anode and/or cathode electrode chambers of the bioreactor (ElMekawy et al. 2014). PhotoMFCs were first examined for light-driven microbial electrogenesis in the 1960s using *Rhodospirillum rubrum* and *Oscillatoria* spp. as biocatalysts (Berk and Canfield 1964). In the 1980s electron mediators were added to anode chambers to produce higher power density (Rosenbaum et al. 2010; Tanaka et al. 1985). In general, phototrophic electrogens are far less electrogenic than heterotrophic electrogens such as *Geobacter*. Photosynthetic microorganisms are widely distributed in nature, specifically in surface waters containing dissolved nutrients and prevalent solar energy exposure. PhotoMFCs may have the potential for removing carbon dioxide from the atmosphere (Cao et al. 2009; De Schamphelaire and Verstraete 2009; Rosenbaum et al. 2010). Figure 18.4 depicts a photoMFC.

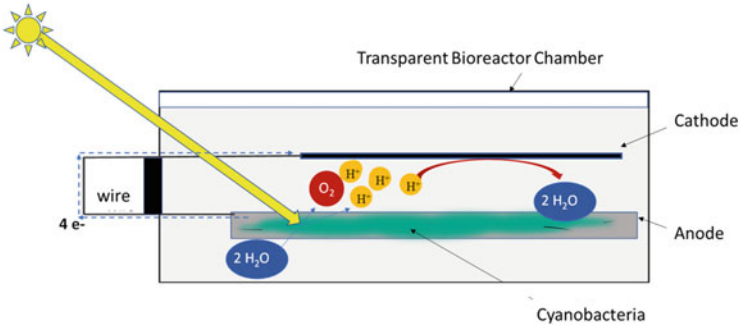

Fig. 18.4 PhotoMFC: A cyanobacterial biofilm grown on an illuminated anode photolyzes H_2O to form oxygen plus protons as electrons are donated to the anode, building a voltage. Electrons flow as DC from the anode to the cathode where electrons reduce oxygen in the presence of protons to reform water

Electrogenic photosynthetic microorganisms create an electrical current by providing water-derived electrons to anode electron acceptors (Pisciotta et al. 2011). By contrast, heterotrophic electrogens, like *Geobacter* species, oxidize organic molecules to yield electrons through respiratory metabolism (Chaudhuri and Lovley 2003). PhotoMFC systems that use oxygenic phototrophs, such as cyanobacteria or microalgae, are sometimes termed bio-photovoltaic (BPV) devices, due to their generation of electricity through light-driven water oxidation (Tschörtner et al. 2019; Qi et al. 2018). PhotoMFCs function using the oxygenic reaction centers of the cyanobacterial photosynthetic apparatus (Tschörtner et al. 2019, Qi et al. 2018). These systems can be set up using either two or three electrodes (Wey et al. 2019). In the dual configuration, the anode and cathode (either in a single chamber or dual chambers) use microorganisms in the anode to transfer electrons through an outside electrical circuit to the cathode (Fischer 2018). Protons arising from water photolysis at the anode are transferred via diffusion through a PEM (if present) to the cathode (Fischer 2018). Since oxygenic phototrophs create O_2 and are not inhibited by it, a PEM is less often used in photoMFCs than in traditional heterotrophic anaerobe-driven MFCs. At the cathode, oxygen is reduced in presence of protons to form water as occurs in a traditional MFC (Fischer 2018). The difference in potential between the anodic and cathodic reactions drives the electron transfer (i.e., electric current) from anode to cathode (Wey et al. 2019). Resistors can be attached to the external portion of the circuit. A voltmeter can then be used to measure the voltage drop across the resistor to calculate the electric current in amps according to Ohm's law. Using a current measurement as a function of external resistance or applied voltage provides the system power generation through a power density curve (Wey et al. 2019). Power generation, normalized in terms of mW/m^2 power density, is illustrated for a diversity of electrogenic microorganisms in Fig. 18.5.

Potentiostats are expensive instruments used to characterize MFCs and photoMFCs in detail using various electrochemical methods. Chronoamperometry

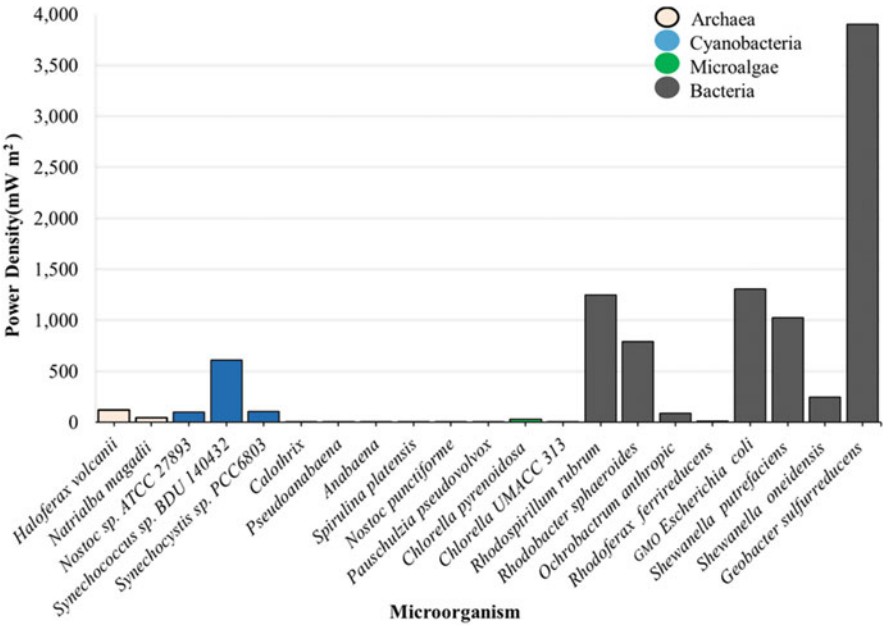

Fig. 18.5 Chart of reported power densities generated by various electrogenic microorganisms. Model electrogen *G. sulfurreducens* demonstrates highest activity. (Sources: Abrevaya et al. 2011, Bombelli et al. 2014, Chaudhuri and Lovley 2003, Cho et al. 2008, Fu et al. 2010, Gomez et al. 2014, Kaushik et al. 2017, Lapinsonnière et al. 2013, Luimstra et al. 2013, Ng et al. 2014, Pisciotta et al. 2010, Qiao et al. 2014, Sekar et al. 2014, Wenzel et al. 2018, Xiang et al. 2009, Xu et al. 2015, Yi et al. 2009, Zuo et al. 2008)

is employed to measure current output over time. This technique allows for the quantification of bioanode electrogenic or biocathode electrotrophic activity in response to different conditions such as temperature or light intensity (Wey et al. 2019). Cyclic voltammetry provides information on redox reactions occurring within BESs, for instance, relating to soluble mediators (Wey et al. 2019). In a three-electrode MFC configuration, a potential can be applied to the working electrode to be precisely monitored in relation to a reference electrode using a potentiostat (Compton and Banks 2018). The reference electrode is typically calomel or Ag/AgCl, and the working electrode is generally a bioanode with electrogenic microorganisms (Wey et al. 2019). Impedance spectroscopy is another potentiostatic technique that can be used to measure internal resistance (Ω) in BESs. Using a three-electrode system can allow for the measurement of anode or cathode impedance (Sekar and Ramasamy 2013). To measure anode impedance in a three-electrode cell, the cathode acts as a counter electrode, the anode is the working electrode, and the reference electrode is placed close to the anode (Sekar and Ramasamy 2013). This setup is similar for measuring cathode impedance, except the cathode is the working electrode, the anode is the counter electrode, and the reference electrode is placed near the cathode (Sekar and Ramasamy 2013).

One established photoMFC design uses a dual-chambered configuration, one anodic chamber and one cathodic chamber separated by a PEM (Gul and Ahmad 2019). Transparent electrodes may be integrated into the design (Wenzel et al. 2018). PhotoMFCs have traditionally used ferricyanide or permanganate as a reduced electron acceptor or platinum as a catalyzer (Qi et al. 2018). These photoMFCs have only been investigated in laboratory studies, however, due to the potential environmental impact of the materials (Jang and Kim 2004; Wei et al. 2012). This environmental concern prompted the use of air cathodes for practical applications (Bosch-Jimenez et al. 2017; Yang et al. 2017a, b). Some studies have proposed the use of single-chamber photoMFCs in recent years as well (Chandra et al. 2015; Lai et al. 2017; Wang et al. 2011). These devices are generally configured in three ways: subcellular photoMFC, cellular photoMFC, and complex cellular photoMFC (Mccormick et al. 2015; Qi et al. 2018). In a subcellular photoMFC, purified elements are directly attached to the surface of the electrode (Qi et al. 2018). Most cellular photoMFCs use living APB species in the anaerobic chamber to generate bioelectricity and are widely used today (Qi et al. 2018). A complex cellular photoMFC may contain producers and consumers, algae, and heterotrophic bacteria or APB (Qi et al. 2018).

Mechanistically, electrogenesis in photoMFCs differs from conventional MFCs in that it is associated with the photosynthetic electron transport chain (P-ETC). More specifically, photosystem II (PSII) mediated photolysis of water, providing a source of electrons (Pisciotta et al. 2011). Electrogenic activity of cyanobacteria is light-dependent, requiring the donation of electrons to extracellular electron acceptors during illumination (Zou et al. 2009). Plastoquinone (PQ) is a crucial component of the P-ETC facilitating electron flow from PSII to cytochrome b6f (Berry et al. 2002). PQ is found in both the cell membrane and the thylakoid membranes and is used by both the respiratory electron transport chain (R-ETC) and P-ETC (Paumann et al. 2005). PQ in its oxidized state can accept electrons from PSII, and subsequent oxidation of PQ donates electrons to cytochrome b6f through photochemical reactions in the z-scheme ETC (Pisciotta et al. 2011). Conditions that cause an over-reduction in the PQ pool, such as high light intensity, allow PQ to donate electrons to cytochrome bd quinol oxidase, providing an alternative electron flow route to the exterior and thereby generation of electric current (Pils and Schmetterer 2001; Pisciotta et al. 2011). To deal with high light intensities, phototrophs have adopted various mechanisms of photoprotection, one being nonphotochemical quenching (NPQ) (Karapetyan 2007). In NPQ, excess light energy is fluorescently dissipated as infrared radiation or heat (Karapetyan 2007). The movement of electrons from the thylakoid to the plasma membrane and then to the exterior of the cell is termed exoelectrogenesis (Hasan et al. 2017). Electrogenic activity with cytochrome-mediated electron transfer to the exterior environment (e.g., anode surface) may be a type of overflow metabolism to protect cells against light-induced cellular damage (Pisciotta et al. 2011). However, even pelagic cyanobacteria generate electricity when illuminated, indicating direct cytochrome and/or nanowire-mediated electron transfer are not the only transfer mechanisms. Shlosberg et al. (2021) recently found that NADPH serves as a secreted electron

mediator in cyanobacteria. Exogenous NADP+ increased photocurrent production by cyanobacteria, showing potential as a nontoxic, water-soluble electron mediator (Shlosberg et al. 2021).

Cyanobacteria have been evaluated in multiple photoMFCs studies to ascertain their electrical power generation capability. Ma et al. (2012) reported that *Synechocystis* PCC-6803 was able to produce a maximum power density of 72.3 mW/m^2 in a dual-chamber model. In another study, Fu et al. (2010) found that *Spirulina platensis* could be used in an open circuit without external organic fuel, obtaining a maximum power density of 6.5 mW/m^2. A novel strain of *Nostoc* sp. ATCC 27893 generated a maximum power density of 35 mW/m^2; however, after 1,4-benzoquinone mediator was added, the maximum power density improved to 100 mW/m^2 (Sekar et al. 2014). Of all known exoelectrogenic microorganisms (photosynthetic or otherwise), *Geobacter* sp. consistently provides the highest power outputs (Wang et al. 2018b; Yi et al. 2009). Ren et al. (2016) reported 5610 mW/m^2 using *Geobacter*-enriched heterotrophic cultures. PhotoMFC power output is at least an order of magnitude lower than such heterotrophic MFCs and not high enough for most practical applications presently. However, efforts are underway to improve output, and photoMFCs could be warranted in remote areas of high solar energy exposure where small instrument powering is needed. On the cathode of MFCs, illuminated cyanobacteria can also play a role by facilitating the O_2 reduction reaction through water photolysis. Reported power generation of various photosynthetic electrogens is reviewed in Table 18.2.

Anoxygenic phototrophic bacteria (APBs) were some of the first microbes investigated, along with cyanobacteria, in photoMFCs by Berk and Canfield (1964). APBs do not generate oxygen as a product and are mainly purple bacteria, green bacteria, and heliobacteria (George et al. 2020). Many APBs are inhibited by oxygen and perform photosynthesis without oxygen formation nor PSII involvement (George et al. 2020). Sulfide, organics, hydrogen, or other electron donors provide reducing equivalents instead of water (Canfield and Farquhar 2012; George et al. 2020; Imhoff 2008). APBs pigment-protein complexes may contain bacteriochlorophyll β (βChl) instead of chlorophyll, carotenoids, primary light-harvesting complex (LH1), peripheral light-harvesting complex (LH2), and reaction centers (RCs) (George et al. 2020). The RC is where light energy is converted into chemical energy, and LH1 and LH2 perform light harvesting and transmission to the RC in anoxygenic photosynthesis (Mascle-Allemand et al. 2010; Sundström et al. 1999). APBs use either type I RCs typically found in phyla *Chlorobi*, *Acidobacteria*, and *Firmicutes* or type II RCs found in species of the phyla *Chloroflexi*, *Proteobacteria*, and *Gemmatimonadetes* (George et al. 2020; Hanada 2016).

Generation of bioelectricity by APBs can be accomplished through the production of electrons by anoxygenic photosynthesis and endogenous respiration or the use of APB-produced hydrogen that subsequently oxidizes on the anode surface, reducing it (Qi et al. 2018). *Rhodopseudomonas* is a commonly used APB at the photoMFC anode although current and power density is generally low (Gomez et al. 2014; Pintucci et al. 2015). Because of the high cost associated with metal catalysts, like platinum, anode metal catalyst usage for MFC applications is likely not feasible

Table 18.2 Power densities of photoMFCs showing representatives of three different categories of photosynthetic microorganisms and anode differences

Group	Organism	Anode	Power density (mW/m^2)	References
Cyanobacteria	*Anabaena* sp.	Au	0.0004	Chiao et al. (2006)
	Synechocystis sp. PCC 6803	Poly-A coated CC	1.47	Zou et al. (2009)
	Synechocystis sp. PCC6803	ITO/CPt	1.2	Bombelli et al. (2011)
	Spirulina platensis	Pt	6.5	Fu et al. (2010)
	Photosynthetic biofilm	Nano-PolyP	5.8	Pisciotta et al. (2010)
	Calothrix	CP-P	0.807	Pisciotta et al. (2010)
	Pseudanabaena	CP-P	0.502	Pisciotta et al. (2010)
	Synechococcus	CP-P	0.472	Pisciotta et al. (2010)
	Anabaena	CP-P	0.453	Pisciotta et al. (2010)
	Nostoc	CP-P	0.414	Pisciotta et al. (2010)
	Nostoc sp. ATCC 27893	CNT/CP	35	Sekar et al. (2014)
	Nostoc sp. ATCC 27893	CNT/CP	100	Sekar et al. (2014)
	Synechocystis sp. PCC6803	InSnBi alloy/Pt	105	Bombelli et al. (2014)
	Synechococcus sp. BDU140432	SF/QD/GNP/GE	610	Kaushik et al. (2017)
Eukaryotic algae	*Chlorella vulgaris*	FTO/CC	24	Thorne et al. (2011)
	Pauschulzia pseudovolvox	PPCP/CC with Pt	6.2	Luimstra et al. (2013)
	Chlorella UMACC 313	ITO-coated glass	0.289	Ng et al. (2014)
	Chlorella pyrenoidosa	GR	30.15	Xu et al. (2015)
	Chlorella sp. UMACC 313	ITO-coated glass	0.289	Ng et al. (2017)
Anoxygenic photosynth. Bacteria	*Rhodobacter sphaeroides*	Pt-coated, CP	790	Cho et al. (2008)
	Rhodopseudomonas sp. with *Thiorhodovibrio* sp.	GP	112.2	Chandra et al. (2015)
	Sediment (SMFC) containing *Rhodopseudomonas* sp.	CF	202.9	Zheng et al. (2017)

(Qi et al. 2018). Biocathodes have become of interest in recent years, and some species may boost O_2 levels facilitating O_2 reduction reaction (Rago et al. 2018; Wang et al. 2018a; Wetser et al. 2017; Qi et al. 2018). The type of substrate used in any MFC is of great importance. APB has complex metabolic pathways and various substrates, including acetate and lactate, may be catabolized (Qi et al. 2018). Because of the versatility of metabolic processes of APB, they are a model microorganism for use in environmental waste bioremediation (George et al. 2020).

18.2.3 Plant Microbial Fuel Cells

Plant microbial fuel cells (plant-MFCs) use plant roots as a direct source of photosynthetically created organic fuel via rhizodeposits for electrochemically active microorganisms at the anode (Moqsud et al. 2015; Schamphelaire et al. 2008; Strik et al. 2008a, 2011). Strik et al. (2011) explored the use of this technology in a plant sediment microbial fuel cell (psMFC) using reed mannagrass at the anode and root exudate for the organic matter for the anodic microorganisms (Moqsud et al. 2015). The results showed that adding additional biomass through plants to the MFC increased the power and current densities generated (Strik et al. 2008a). These systems have since been designed to work as ecological systems in constructed wetland MFC systems (Helder et al. 2010; Xu et al. 2015; Yadav et al. 2012). Interest in plant-MFCs continues to rise due to the various applications including bioelectricity, pollution removal, and environmental remediation potential (Deng et al. 2011; Moqsud et al. 2015; Nitisoravut and Regmi 2017). MFCs of this type involve the use of living plants, a supportive environment with an electrically conducive anode, appropriate substrate, and a cathode (Kabutey et al. 2019). The anode of a plant-MFC is incorporated into the substrate, while the cathode is added to water or used as an air cathode (Cheng and Liu 2014; Nitisoravut and Regmi 2017). During substrate oxidation by the anodic microorganisms, chemical energy from the substrate organic matter becomes converted to protons, electrons, and CO_2 (Kabutey et al. 2019). Anodic electrons are transported to the cathode through an electrical circuit, while protons travel through the matrix to the cathode (Kabutey et al. 2019). The terminal electron acceptor oxygen is reduced with protons and electrons at the cathode to form water and generate bioelectricity (Kokabian and Gude 2013; Strik et al. 2008a). Configurations of plant-MFCs include single- and dual-chamber designs (Deng et al. 2011). In a single-chamber plant-MFC type, there exist a membrane-less anode and no cathode chamber (Deng et al. 2011). In the dual-chamber orientation, there is an anodic and cathodic chamber separated by a PEM, similar to a MFC (Kabutey et al. 2019; Rabaey et al. 2003).

Bioelectricity is the primary application originally investigated in plant-MFCs; however, they are now considered for additional uses, similar to MFCs and photoMFCs (Nitisoravut and Regmi 2017; Strik et al. 2008b). This technology could be used for bioelectricity generation in wetlands or wastelands that are not conducive for agriculture or incorporated for use on agricultural lands without impacting food production (Helder et al. 2010; Wetser et al. 2015). Indoor growing

facilities could use plant-MFCs for bioelectricity and air quality maintenance, potentially adapting to an ecologically sustainable system (Kabutey et al. 2019; Helder et al. 2013; Tapia et al. 2017). These systems have also been examined for their use in wastewater treatment and for roof gardens to lower the effects of urban development (Klaisongkram and Holasut 2014; Nitisoravut and Regmi 2017; Skinner 2006).

Plants are the most critical component in a plant-MFC, which has been investigated as a pollution indicator and remediator (Kabutey et al. 2019). Wetland plants are common biocathodes due to their high tolerance to water and ease of O_2 intake from the atmosphere into their roots (Kothapalli 2013). Any plant used in a plant-MFC must convert solar energy into usable chemicals without the requirement of harvesting the plant (Halan et al. 2017; Strik et al. 2008a). When the plants photosynthesize, organic matter is produced, which can then be used by the microorganisms in the rhizosphere (Narula 2017; Strik et al. 2008a). Microorganisms in the rhizosphere are another important component of plant-MFCs. The rhizosphere is the region directly surrounding the roots and root surface of a plant (Kabutey et al. 2019). This area of the soil supports a large variety of different species and activities, along with a surface for microbial attachment (Bakker et al. 2013; Foster et al. 1984; Kabutey et al. 2019). Microorganisms utilize substrate in the rhizosphere through direct electron transfer or indirect (mediated) electron transfer (Nitisoravut and Regmi 2017). The symbiotic relationship rhizosphere microbes establish with plants is due in part to the organic matter that serves as an energy source for the microbes, while the microbial reactions increase the plants uptake of key elements (Moulin et al. 2001; Nitisoravut and Regmi 2017).

Soil is an important factor in the design of most conventional plant-MFCs. Soils can differ vastly by structure, texture, and pH, along with available usable nitrogen, all affecting a soil microbial community (Frey et al. 2004; Lauber et al. 2009; Nitisoravut and Regmi 2017; Sessitsch et al. 2001; Wakelin et al. 2008). Chemical decomposition in the soil can produce electrons that play a role in plant-MFC bioelectricity generation since constant redox reactions occur within the soil (Nitisoravut and Regmi 2017; Vepraskas and Faulkner 2001). The supporting matrix is an important factor when designing a plant-MFC because it affects the internal resistance of the system by affecting proton movement between the electrodes along with root exudate diffusion at the anode (Cheng and Liu 2014; Takanezawa et al. 2010). Some examples of supporting matrix in plant-MFC include paddy, wetland soils, garden soils, flooded soils, and different sediments, among others (Cheng and Liu 2014; Helder et al. 2010; Kabutey et al. 2019). The basic mechanism for a plant-MFC is shown in Fig. 18.6.

Soil-less aquatic plant microbial fuel cells (SLAP-MFCs) have recently been devised with potential for biological electricity production coupled with nutrient pollutant removal from water bodies. *Lemna minor* (duckweed) is a small, protein-rich aquatic plant that floats atop the surface of the water and is generally found in calm, fresh to brackish waters (Hubenova and Mitov 2012). Related *Lemna minuta* can be used to convert solar energy into electricity and successfully acts as a biocatalyst (Hubenova and Mitov 2015). Electrical output (380 ± 19 mW/m^2)

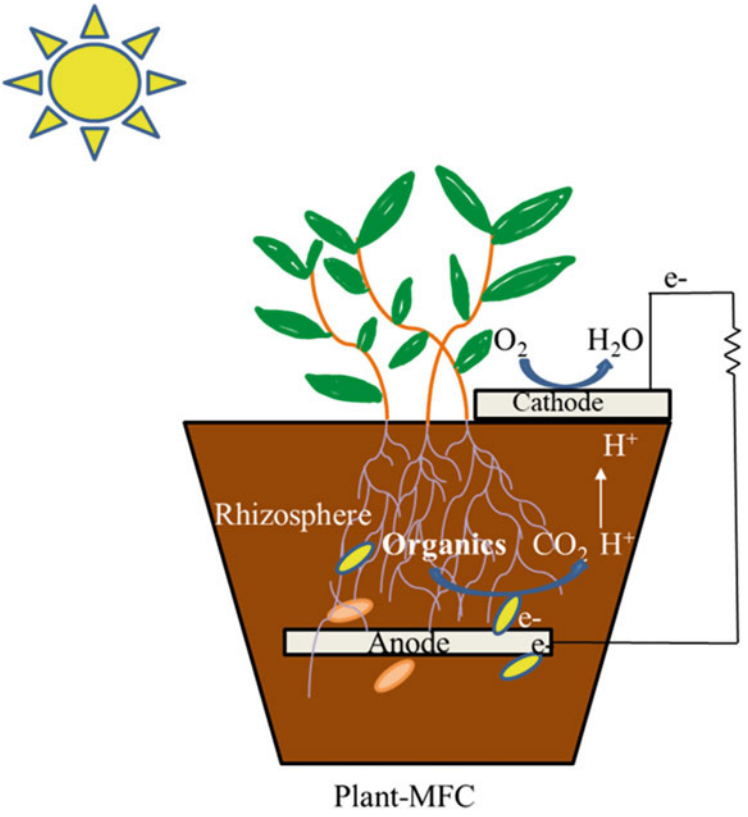

Fig. 18.6 Plant microbial fuel cell (Plant-MFC). Plant-MFC uses solar energy and rhizosphere microbial metabolism to generate bioelectricity. Photosynthesized organics released from the plant roots support microbial catabolism of organics into CO_2, protons (H^+), and electrons. Electrons deposited to the anode by electrogenic bacteria are channeled through an external circuit to the cathode. H^+ diffuses through the soil to the cathode, where the reduction of oxygen to form H_2O occurs

obtained by Hubenova and Mitov (2012, 2015) was dependent on both illumination and temperature. Daytime high current densities attributed to light-dependent reactions showing a 10–20% higher yield than nighttime and are associated with a *Lemna*-produced endogenous mediator (Hubenova and Mitov 2012, 2015). Hubenova et al. (2018) suggest that absorption of red light by duckweed has a role in photosynthetic reactions and extracellular electron transfer and plant cytochromes play a role in extracellular electron transport, similar to *Geobacter*-based MFCs (Hubenova et al. 2018). Duckweed's use in power generation through SLAP-MFCs could prove a useful tool in remote locations where solar energy and water are plentiful.

Lemna spp. incorporate a survival mechanism in which it forms turions, compact fronds that contain starch grains, for withstanding unfavorable environmental

conditions or lifecycle dormancy stages (Landolt 1986; Verma and Suthar 2015). Conceivably, this starch could be extracted for saccharification followed by fermentative bioethanol production. Duckweed is highly productive and accounts for up to 100 tons of dry matter/ha/year of biomass in some areas (Cao et al. 2018). Though small, this plant has many current uses, including food or feed additives for livestock and improvement of water quality (El-Shafai et al. 2007; Lahive et al. 2011; Muradov et al. 2014; Stout and Nüsslein 2010; Ziegler et al. 2016). Uses for duckweed relate to (1) its use in electrical generation, (2) its potential for improving water quality, and (3) its potential as a valuable food or feedstock for bio-based energy operations such as anaerobic digestion (Hubenova and Mitov 2012; Lahive et al. 2011; Muradov et al. 2014; Stout and Nüsslein 2010; Verma and Suthar 2015; Ziegler et al. 2016). Further, it has potential applications as biofuel in starch to ethanol conversion or wastewater treatment (El-Shafai et al. 2007; Hubenova and Mitov 2012; Muradov et al. 2014; Stout and Nüsslein 2010). These nitrogen- and protein-rich aquatic macrophytes can be used as organic fertilizer when applied to fields (Ahmad et al. 1990). SLAP-MFCs could have utility for enhancing water quality by removing toxic nutrients and heavy metals from the water, all while regulating oxygen balance (Lahive et al. 2011; Muradov et al. 2014; Ziegler et al. 2016; Kumar et al. 2021). Production of duckweed is influenced by nutrients in runoff, light intensity, photoperiod, and temperature (Lasfar et al. 2007; Muradov et al. 2014; Yin et al. 2015).

18.3 Microbial Electrosynthesis

Microbes, like all organisms, require energy to assemble the chemical building blocks needed for growth and replication. Conventional biology states two forms of energy support life. On Earth's surface, light from the Sun drives photosynthesis of CO_2 into organic carbon in photoautotrophs like plants and algae. Solar energy similarly provides for N_2 fixation from the atmosphere into amino acids in diazotrophic microorganisms. This can occur directly, as happens in certain species of cyanobacteria, or indirectly as is the nature of the diazotrophic symbiosis between leguminous plants and their Gram-negative root rhizobia endosymbionts (Petersen and Yuen 2020). Fixed organics in the biomass of these primary producers are then consumed and preyed upon by higher trophic levels to support the entire ecosystems. Organic chemical energy in this way powers growth and metabolism in herbivores, humans, and other organoheterotrophs. Conversely, lithoautotrophic prokaryotes, like the endosymbionts within giant marine *Riftia* tubeworms, gain energy to support Calvin cycle anabolism through enzyme-mediated oxidation of reduced inorganic chemicals like H_2S (Hinzke et al. 2019). Chemosynthesis by lithoautotrophic primary producers can in this way support ecosystems in total darkness. Challenging the time-honored dogma that only light or reduced chemicals power living organisms is the relatively recent discovery that electrical energy too can support autotrophic growth of a group of microbes called electroautotrophs or electrotrophs (Lovley 2010).

In sunlit regions, photosynthesis is widely used to grow biofuel feedstocks, like sugarcane, corn, or algae, from CO_2 plus light. However, photosynthetic organisms are far less efficient at capturing solar energy than modern photovoltaic panels, necessitating large crop cultivation areas (Giddings et al. 2015). Even the most efficient plants store solar energy at below 1% efficiency (Dogutan and Nocera 2019). Modern solar panels are about 25% efficient at converting light to solar electricity. Microbial electrosynthesis is a biotechnological process that seeks to improve the efficiency of primary production by harnessing electrotrophs to catalyze the fixation of CO_2 into organic matter by using electrons supplied from the cathode of a BES (Jung et al. 2020; Nevin et al. 2010). In contrast to MFCs, in which electrogens are grown on an anode and donate electrons to the electrode, electrotrophs use the BES cathode as their source of electrons. Electrotrophs thereby consume current to store electrical energy in chemical bonds via the electrosynthesis of molecules (Nevin et al. 2010). While interest in bioanodes has greatly increased over the past 20 years (Park and Zeikus 2000), sustained research into electroautotrophy and electrosynthesis is hardly half as old with far less known about the fundamental process.

Electrosynthetic BESs have been created which connect biocathode electrotrophs to fix CO_2 into organic molecules like acetate or bioelectrochemically formed biofuels called electrofuels (Nevin et al. 2010; Zaybak et al. 2013). Not all biocathodes are electrosynthetic nor strictly anaerobic; microbial biocathodes were developed in MFCs to facilitate oxygen or nitrate reduction reactions (Cha et al. 2009). Biocathodes use diverse microorganisms at the cathode to perform catalysis and improve electrical current and power use in the whole system (Watanabe 2008). Using a biocathode can eliminate the need for mediators in the cathode region and can lower cost while simplifying BES configuration (Watanabe 2008). One application for biocathodes from the electron delivery standpoint is the acceleration of microbial metabolism to remove reaction by-products favoring the generation of usable products (He and Angenent 2006). BES cathode materials include carbon materials such as carbon fiber, carbon paper, carbon brush, carbon felt, graphite, Pt, and reticulated vitreous carbon (Chen et al. 2008; Nandy and Kundu 2018; Park and Zeikus 2002). Graphite is commonly used for electrosynthetic biocathodes and to study electrotrophs. Microbial electrosynthesis refers to the microbially mediated, electrically driven fixation of CO_2 into organic material (Nevin et al. 2010). Both direct and indirect forms of electrosynthesis exist although direct processes are inherently simpler and more efficient. To support indirect electrosynthesis, hydrogen produced at the cathode or even in a separate reactor may serve as electron carrier (Agostino et al. 2020). Alternatively, soluble chemical mediators, such as neutral red, can be added to support indirect microbial electrosynthesis (Jeon et al. 2011). Inorganic electron carriers other than H_2 can also be used to support growth. Kinsel and Umbreit may have been the first to indirectly cultivate an electrotroph when they used dissolved iron at low pH to shuttle cathode-derived electrons to autotrophically growing *Acidithiobacillus ferrooxidans*, formerly *Ferrobacillus ferrooxidans* (Kinsel and Umbreit 1964). Widely used in biomining, such extremely acidophilic chemolithoautotrophic bacteria rely on the Calvin cycle to fix CO_2 with reducing

power (i.e., electrons) provided by dissolved ferrous iron. In their studies, the cathode replaced the ore that is normally oxidized to support growth and metabolism.

18.3.1 Electrotrophic Microorganisms at Biocathode

Pure cultures of direct electrotrophic bacteria, such as *Sporomusa ovata*, as well as mixed electrotrophic communities have been electrotrophically cultivated in minimal media with CO_2 as the carbon source without mediators (Nevin et al. 2010; Pisciotta et al. 2012). Methanogenic and acetogenic prokaryotes were found to possess the capacity for electrotrophic growth and metabolism, representing both archaea and bacteria, respectively (Cheng et al. 2009; Nevin et al. 2010). Some of the best-studied electrotrophic bacteria are acetogenic members of the genera *Clostridium*, *Moorella*, or *Sporomusa* (Chen et al. 2018). Drawbacks associated with microbial electrosynthesis include slow start-up rates and low current density, particularly for pure cultures (Logan et al. 2019). Elucidation of the metabolic pathways and physiological mechanisms that provide for this unusual form of autotrophic growth could provide rational options for process optimization through targeted genetic modification (Jung et al. 2020; Zaybak et al. 2018). Identification of novel electrotrophs from nature is another interest area with both practical applied uses and fundamental ecological and biochemical aspects.

Metabolically, the Wood-Ljungdahl pathway provides for CO_2 fixation in most electroautotrophic microbes discovered to date. The Wood-Ljungdahl pathway, also known as the reductive acetyl-CoA pathway, is the most efficient and direct of the six known autotrophic pathways or cycles that support life (Song et al. 2020). Prokaryotes that rely on this pathway use H_2 as the high-energy electron donor and CO_2 (or CO) as their carbon source (Wood et al. 1986). Oxidation of H_2 is catalyzed by hydrogenases, and electrons are transferred to metal-containing pathway enzymes used to reduce one molecule of CO_2 to form a methyl group and another CO_2 to form the carboxyl group of acetate. Some of the acetates is secreted, while some is integrated into growing biomass. The central enzyme complex required for Wood-Ljungdahl pathway-mediated CO_2 fixation is carbon monoxide (CO) dehydrogenase (CODH)/acetyl coenzyme A (acetyl-CoA) synthase (Carlson and Papoutsakis 2017). Variant types of CODH/ACS complexes are found in other groups of autotrophic microbes, including some sulfate-reducing bacteria (SRBs). Genetic screening for CODH/ACS is a useful method for the preliminary identification of novel electrotrophs. This prescreening method was recently used for microbes such as *Desulfobacterium autotrophicum* HRM2 (Zaybak et al. 2018).

Physiologic mechanisms employed and deployed by electrotrophs to harness direct electric current from cathodes for fixation of inorganic carbon are the subject of ongoing research. At applied cathode potentials below −600 mV vs. standard hydrogen electrode (SHE), H_2 begins to be produced abiotically by plain graphite electrodes which are the type of electrodes normally used to grow and study electrotrophs (Nevin et al. 2010). At neutral pH, H_2 production theoretically can

commence at −610 mV (Agostino et al. 2020). Accordingly, studies aimed at investigating direct electron uptake by electrotrophs use applied voltages of −600 mV or above (i.e., more positive) to ensure that electrons are not indirectly delivered to the microbe via diffusion of H_2 (Nevin et al. 2010; Pisciotta et al. 2012). Various cell surface structures facilitate direct electron uptake from cathodes by electrotrophs.

Membrane-associated, multi-heme cytochromes (MHCs) provide conductive electron transfer routes enabling electrons to bridge both membranes and periplasm of Gram-negative SRBs. MHCs in *Desulfovibrio ferrophilus* IS5, a Gram-negative within the order *Desulfovibrionales*, afford access to internal metabolism and reduction of cytoplasmic electron carriers like NAD+ (Deng et al. 2018). Cyclic voltammetry analysis of the electroautotrophic SRB *Db. autotrophicum* HRM2 (order *Desulfobacterales*) further suggests MHCs are involved in electron uptake from cathodes across disparate orders of Gram-negative bacteria (Zaybak et al. 2018). Physiological differences pertaining to the route of electron uptake are clearly apparent in Gram-positive autotrophic SRBs. *Desulfosporosinus orientis* (order *Clostridiales*) is deficient in MHCs, has a thick peptidoglycan cell wall, and requires cathode potentials more negative than −600 mV for electron uptake and autotrophic growth demonstrating H_2 as an indirect electron transfer mediator. The thick cell wall of Gram-positive bacteria presents a physiological challenge to the efficient uptake of electrons. Indeed, administration of sublethal penicillin to reduce the cell wall of the known electrotroph *Moorella thermoautotrophica* resulted in approximately a doubling of electron uptake and electrosynthetic formed acetate with an improvement in coulombic efficiency (Chen et al. 2018). *Clostridium pasteurianum* is a Gram-positive bacterium that is capable of direct electron uptake from cathodes (Choi et al. 2014). Much less is known about how Gram-positive bacteria exchange electrons with electrodes than is the case for Gram-negatives (Paquete 2020). Some Gram-negative bacteria and cyanobacteria synthesize pili-like appendages to aid with electron exchange. Microbial nanowires are thin, elongate membrane projections that support microbe-electrode electron exchange that was first detected in anode-associated electrogens (Gorby et al. 2006). Microbial nanowires featuring MHC enrichment appear to increase surface area contact between the cathodes and some species electrotrophs (Deng et al. 2018). Genetic knockout of MHC genes in *Methanosarcinales* did not prevent uptake of electrons from a cathode at −400 mV (Yee and Rotaru 2020). Therefore, additional electron transfer mechanisms appear to be at work in biocathode-associated species. Secreted hydrogenases may also facilitate the transfer of electrons from cathode to cells (Deutzmann et al. 2015; Reiner et al. 2020). Mechanisms of cellular electron uptake from cathodes are summarized in Fig. 18.7.

Electrons must be sourced from the anode side to supported electrotroph growth and metabolism at the cathode side. Electrons may be donated through anode-mediated electrolysis of inorganic molecules, such as water. Unfortunately, O_2 produced during the energetically unfavorable water splitting reaction is inhibitory to many anaerobic electrotrophs, necessitating an expensive PEM and dual-chambered design. Sulfide has been used as an alternative inorganic electron

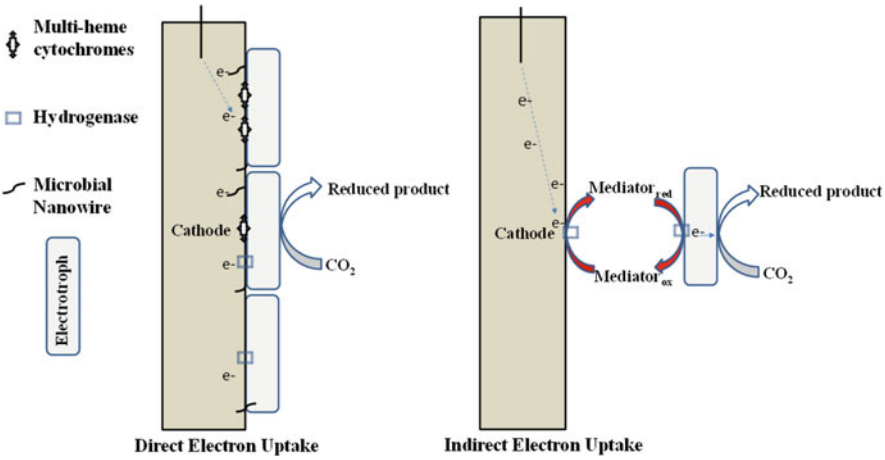

Fig. 18.7 Physiological mechanisms of direct (left) and indirect (right) electron uptake from biocathodes

donor to anoxygenically supply reducing equivalents to support electrotrophs (Gong et al. 2012). Oxidation of sacrificial scrap metal anodes can source copious electrons with concurrent anode valorization into metal-containing nanoparticles (Pisciotta 2019). Alternatively, a bioanode featuring heterotrophic electrogens like *Geobacter* spp. can be utilized to oxidize organic molecules as the electron source. Since electrogens speed waste treatment as they contribute to the BESs voltage, sourcing of electrons from organic waste is an attractive option to support microbial electrosynthesis (Nevin et al. 2010; Pisciotta 2014). In either case, as electrons travel through an external circuit from the anode to the cathode, voltage is boosted by an external power source, such as a photovoltaic panel. A benefit of electrosynthesis over photosynthesis is that sunlight is not required since wind power, hydropower, or other sources of DC can be used. Microbes can be cultivated at high density and harvesting from reactors is simpler than harvesting field crops.

Products of electrosynthetic systems are typically small, secreted organic molecules. Efforts have been rigorously focused on the efficient electrosynthesis of biofuel molecules. This is because the US Department of Energy (DOE) supported the initial development of this field of microbiology through its Advanced Research Projects Agency-Energy (ARPA-E) multimillion-dollar Electrofuels Program from 2010 to 2012. Alcohol biofuels ethanol and butanol were synthesized by a mixed biocathode culture at -400 mV with -34 ± 4 mA/m^2 electron uptake, although the primary product was acetate (Zaybak et al. 2013). Indirect electron delivery also supports alcohol electrofuel production, and both ethanol and butanol were produced in an autotrophic *Clostridium*-dominated mixed biocathode culture operated at -800 mV (Ganigué et al. 2015). Propanol was additionally detected in a neutral red-mediated system when applied voltages to -2000 mV were applied to a mixed culture obtained from a digester (Kondaveeti and Min 2015). In general, the

more negative the applied voltage, the lower the efficiency of product production. Ideally, the high current density at an applied potential near the onset point of H_2 formation (−610 mV) could enable copious, efficient direct product electrosynthesis. Understanding the microbial physiology and metabolism of electrotrophs can aid with this. Genetic modification can further facilitate the creation of industrial production strains. Electrodiazotrophy by electrotrophs can serve as a source of protein from the electrically directed, microbial fixation of atmospheric nitrogen.

Challenges associated with electrosynthetic production systems primarily relate to their slow start-up and, in the case of indirectly mediated electron transfer, diminished efficiency compared to direct electron delivery (Izadi et al. 2020). Electrotrophs also tend to form thinner, less robust biofilms than electrogens (Zaybak et al. 2018). Improvements in electrodes and biocompatible electrode-coating materials are one way to elegantly enhance both biofilm development and uptake current density. Just as the use of a nanostructured polypyrrole coating on a bioanode increased power output around fivefold in a photoMFC (Pisciotta et al. 2010), use of polypyrrole conductive polymer on a biocathode recently improved electron uptake and resulted in 3–6 times more electrosynthetic acetate production (Luo et al. 2020). Gas diffusion biocathodes have been developed which can improve electrosynthetic rates (Bajracharya et al. 2016). Coupling biocompatible electrode coatings like nanostructured polypyrrole with gas diffusion cathode configurations can provide synergistic performance improvements by 3D printing biocathodes with cells. A porous electrode that integrated carbon nanotubes in honeycomb-like surface configuration recently demonstrated increased electron transfer rate and elevated coulombic efficiency during acetate electrosynthesis (Flexer and Jourdin 2020). While direct electrosynthesis is considered the most efficient, indirect H_2 electron delivery can be useful to foster initial biofilm growth and development to accelerate start-up. Biofilm development can be enhanced using H_2 at −1000 mV, although at the expense of efficiency (Izadi et al. 2020). As the cost of photovoltaics decreases, the need to rigorously optimize applied potential and electrical efficiency diminishes somewhat as industrial focus shifts to the value of product and rate of production. At applied cathode potentials, more negative than −600 mV H_2 is produced and can serve as indirect elector carrier to electrotrophs, or the H_2 itself can be collected as a product. Production of H_2 at the cathode of an electrically boosted BES is called electrohydrogenesis.

18.3.2 Electrohydrogenesis

Hydrogen has been touted as a near-perfect fuel for decades, if not centuries. Irish alchemist Robert Boyle in 1671 was the first person to conclusively generate hydrogen by dissolving metals in hydrochloric acid; however, it was not until 1766 that Henry Cavendish chemically characterized this “inflammable air” as hydrogen gas (George and Agarwal 2010). The name hydrogen was likely conferred because it resulted in water (i.e., *hydro*) generation upon combustion as hydrogen

combined chemically with atmospheric oxygen. The formation of a harmless, even useful end product is widely recognized as one of the major, environmental benefits of hydrogen as a fuel for the future. It is a clean-burning fuel, the combustion of which releases no greenhouse gasses. Hydrogen is an adaptable storehouse of energy that can power ICE or even jet engines directly or can be chemically oxidized on fuel cell anodes to generate direct current (DC) electricity with high efficiency (Baroutaji et al. 2019; White et al. 2006).

Hydrogen does not require rare or expensive feedstocks for its production. Simple water can be electrolyzed to form H_2 or it can be produced from organic waste by various microbes. Hydrogen can thus be generated via a wide range of biological, chemical, and physical methods. The high degree of flexibility is a key draw of hydrogen. While hydrogen has several benefits, it also suffers from significant drawbacks that have, to date, limited its adoption to certain niche applications. For instance, its high energy density can be a liability since H_2 is extremely explosive as was infamously recorded during the 1937 Hindenburg airship disaster. Hydrogen can spontaneously combust in the presence of oxygen under certain circumstances (Postnikov et al. 2016). Hydrogen gas, as the smallest molecule, also easily escapes many containers and can chemically react with certain materials. Due to its low density, hydrogen must generally be compressed to accumulate significant energy in workable volumes for fuel tanks. This hydrogen compression requires a significant input of energy, thereby limiting overall efficiency. Hydrogen storage, particularly in vehicles, is one of the main stumbling blocks to its broader adoption as a transportation fuel (Joghee et al. 2015). This is partly due to the lack of significant distribution infrastructure for hydrogen-powered vehicles. Despite the drawbacks associated with hydrogen, renewed interest in "green hydrogen" as a possible solution to fossil fuels and issues related to renewable energy storage is spurring research into H_2 production from renewable sources (Velazquez Abad and Dodds 2020). Hydrogen can be produced by chemical, physical, or biological means with microbial production offering the added benefit of organic waste energy treatment. Microbiologically, hydrogen can be produced via three well-known routes: dark fermentation, photofermentation, and biophotolysis. Certain electrically active microbes are involved in a fourth, more recently discovered, electrosynthetic method for producing hydrogen called electrohydrogenesis (Cheng and Logan 2007).

Steam reformation of natural gas-derived methane is an inexpensive route by which most hydrogen is produced at industrial levels today. While the water gas shift reaction can be used to further enhance the amount H_2 formed, reliance on natural gas as feedstock for hydrogen production is unsustainable (Cui et al. 2019). If hydrogen production can be economically linked to waste treatment, the cost of both waste treatment and renewable H_2 generation might be reduced. In 2007 Cheng and Logan discovered that the small roughly -300 mV potential generated by electrogenic anode bacteria in a BES can be boosted using an external power supply to form hydrogen gas with greater efficiency that is possible by water electrolysis and no requisite platinum catalyst (Cheng and Logan 2007). This process, termed electrohydrogenesis, is attractive in that it treats organic wastes and can store renewable wind, solar, or hydropower in the form of clean H_2 gas. Both single-

and double-chambered MECs are effective for H_2 production since O_2 is not formed at the anode (Call and Logan 2008). To help address climate change, there is a growing demand for "green hydrogen" produced without the use of fossil fuels. Challenges include slow reactor start-up times, limited current density, possible inhibition due to waste stream toxins, and consumption of the more valuable hydrogen product by hydrogenotrophic methanogens enriched inside the reactor (Lee et al. 2009; Pisciotta et al. 2012). Since methanogenic archaea are extremely susceptible to O_2, periodic low-level oxygen exposure could help limit methanogens but may also harm beneficial anaerobes. Administration of the peptaibol alamethicin can selectively inhibit methanogens in BESs (Zhu et al. 2015).

Metagenomic studies of hydrogen-producing MECs indicate that electrode polarization enriches hydrogen-producing *Firmicutes* (Arunasri et al. 2016). Hydrogenases appear to facilitate electron uptake by such biocathode-associated species (Jourdin et al. 2015; Reiner et al. 2020). Gene expression analysis of bacteria recovered from MECs indicates upregulation of bidirectional Hox-type hydrogenases associated with cytoplasmic hydrogen formation (Croese et al. 2014). This suggests hydrogenases may provide a conduit for electron uptake on biocathodes and a means of generating H_2. Secretion of extracellular hydrogenases that associate with the cathode could facilitate electron transfer in some species (Deutzmann et al. 2015). Aside from electrosynthesis, the H_2 produced by MECs can be put to work directly on-site for a variety of chemical or energy storage applications. For instance, carbon capture of CO_2 as an inorganic mineral precipitate is possible through the use of microbial reverse-electrodialysis electrolysis and chemical-production cells (Zhu et al. 2014). A MEC used to treat organic waste in wastewater while it stores solar energy in the form of "green" hydrogen gas is depicted in Fig. 18.8.

18.3.3 Electromethanogenesis for Anaerobic Digestion

Methane gas (CH_4) is the smallest hydrocarbon fuel, but it is a critically important energy source in both developing and industrialized nations (Ye 2020). Most of the methane combusted for centralized electricity generation and more distributed applications like building heating, street lighting, and household cooking is a component of natural gas. Around 3.7 trillion cubic meters of natural gas was consumed in 2017 which is projected to grow to 4.9 trillion cubic meters by 2040 when natural gas will provide a quarter of all energy used by humans (Ye 2020). Methane makes up around 90% of natural gas, depending on the good source, with decreasing amount of C2 ethane, C3 propane, C4 butane, CO_2, and condensates (Ponraj and Borah 2020). Hydrogen sulfide (H_2S) may also be present in natural gas and results largely from the anaerobic respiration of thermophilic sulfate-reducing bacteria (SRB) deep within the geologic petroleum formation (Kniemeyer et al. 2007). Reactive sulfur species metabolized by SRBs can facilitate pipeline corrosion through a process called microbial influenced corrosion (MIC) (An et al. 2016).

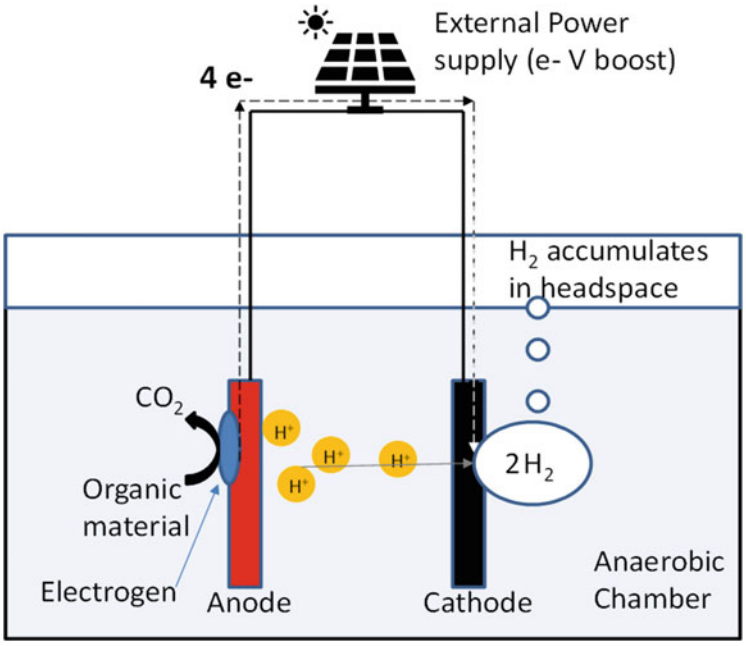

Fig. 18.8 Single-chamber anaerobic MEC. Electrogenic microbes on the anode catabolize organics, generating CO_2 and protons as electrons are donated to the anode, building up a voltage that flows as DC to the cathode. Electrons exit the cathode and reduce protons generating end product H_2. The input energy is supplied by an external solar panel for needed voltage boost since electrohydrogenesis is not spontaneous. H_2 represents stored, renewable solar plus waste energy

Natural gas is a nonrenewable resource that contributes to climate change and is recovered, often as a by-product, from petroleum deposits (Gvakharia et al. 2017). Advances in mining technologies over the past two decades, such as horizontal drilling and hydraulic fracture mining, have made accessible vast reserves of natural gas in deposits including the Marcellus Shale formation that extends through much of western Pennsylvania and the Permian formation in Texas (Hildenbrand et al. 2016; Rahm et al. 2013). Consequently, the price of methane has declined significantly over the past 20 years. At the turn of the millennium, expensive natural gas was deployed in electricity generation to meet peak summertime demand. Due to its price drop, if not environmental cost, natural gas methane is now used for baseload electricity generation since it is now cheaper than coal and releases few pollutants (Liang et al. 2012). Natural gas can be processed to more transportable compressed natural gas (CNG) or even liquified natural gas (LNG) for transcontinental delivery, but this can be economical only a large centralized processing terminal (Economides et al. 2006). Many communities have safety concerns regarding natural gas pipelines and environmental questions pertaining to the use of local hydraulic fracture technologies (Fasola et al. 2019).

Methane is an industrial and agricultural greenhouse gas that is more potent, from a warming standpoint, than carbon dioxide. This is due largely to the fact that methane has a 21.8-fold greater infrared absorptivity compared to CO_2 (Hill et al. 2016; Whiting and Chanton 2001). Although less CH_4 is released annually than CO_2, methane is the second most important emitted greenhouse gas behind carbon dioxide and ahead of nitrous oxide (Shakoor et al. 2020). Methane has a shorter atmospheric half-life than carbon dioxide so reductions in methane emissions can have a relatively rapid beneficial influence on climate (Montzka et al. 2011). In some oil well fields, such as the Bakken oil field of North Dakota, large amounts of natural gas are flared off because its capture and distribution are economically challenging from remote field sites (Gvakharia et al. 2017). Flaring natural gas at the wellhead is a widely used means of diminishing methane's immediate environmental impact but one that is energetically wasteful (Caulton et al. 2014). Flaring is often an incomplete process that releases respiratory irritants and potential carcinogens downwind such as soot and carbon black particulates (McEwen and Johnson 2012). The CO_2 generated also contributes to warming, though to a lesser extent than methane. Unburnt methane from natural gas is introduced directly into the atmosphere in increasing amounts from incomplete wellhead combustion, leaky petroleum industry wells, and pipelines (Gvakharia et al. 2017). The amount of methane leaked from natural gas distribution lines has previously been measured at around five times higher than the EPA limits (Weller et al. 2020). Methane released to the atmosphere from natural sources is of growing concern. Arctic permafrost has been identified as yet another source of increasing atmospheric methane flux in areas like East Siberia (Masyagina and Menyailo 2020). Nations lacking domestic energy and gas reserves on land are turning to vast coastal methane hydrate deposits where, along the continental shelf, abundant methane is sequestered on the seafloor as icelike methane clathrates. In 2013 and 2017, Japan successfully recovered submarine methane hydrates from the seafloor off Honshu (Yamamoto et al. 2019). The effect of seabed methane mining on coastal ecosystems has yet to be thoroughly studied. Production of renewable biologically produced methane is possible and can be enhanced bioelectrochemically (Cheng et al. 2009; Xu et al. 2014).

Under an anaerobic condition in natural settings, such as in swamps, sediments, or the gastrointestinal tract of animals, methane is produced microbiologically through a process called anaerobic digestion. Italian inventor of the electrochemical battery, or voltaic pile, Alessandro Volta was the first to describe the combustible nature of methane. In 1776 Volta collected bubbles of gas rising up from a swamp and discovered that it was highly flammable (Gijzen 2002). This "inflammable air" from the marshes, as it was initially known, was subsequently shown to contain methane, although it took another century before the process by which it formed was put to use industrially in wastewater treatment with attendant biogas formation (Gijzen 2002). This methane was produced from the decomposition of organic material mediated by complex communities of microorganisms. When organic waste is sealed inside an air-impermeable container, termed an anaerobic digester, O_2 is readily consumed, and anaerobes sequentially catabolize the waste. The gas produced and released and accumulated in the anaerobic digester headspace is called

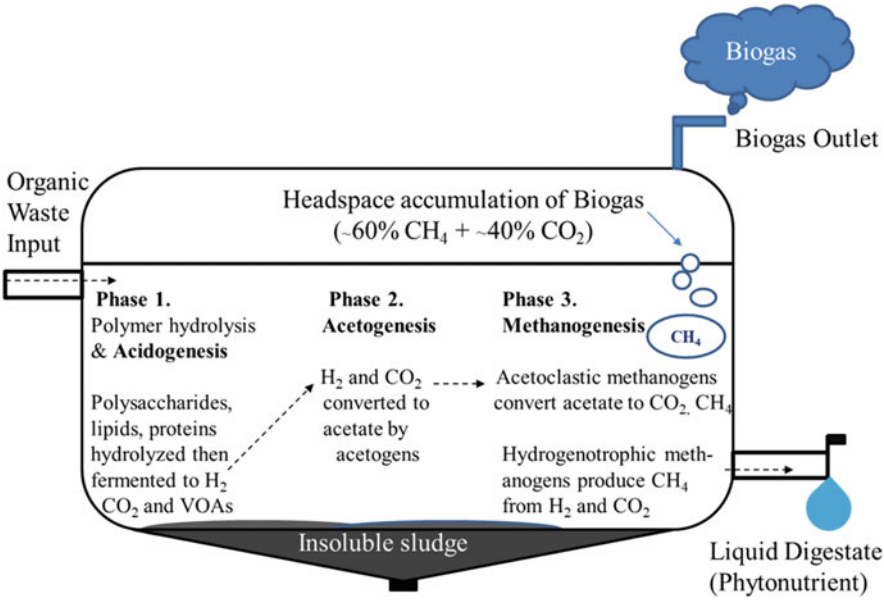

Fig. 18.9 Anaerobic digester showing stages of biogas formation, microbial populations involved, substrates consumed, and metabolic products produced. In stage 1, hydrolysis and fermentative acidification, of large polymeric organic biomass in waste (e.g., proteins, lipids, and polysaccharides), occurs and is the rate-limiting step of anaerobic digestion and results in the formation of CO_2, H_2, and volatile organic acids and is therefore referred to as acidogenesis. In stage 2 (acetogenesis), acetogenic bacteria consume the H_2 and CO_2 and release acetate. In stage 3 (methanogenesis), methanogenic archaea consume acetate, H_2, and CO_2 and release methane gas (CH_4)

biogas. Biogas is usually only 61–64% methane and around 36–38% CO_2 with a trace amount of ammonia and/or hydrogen sulfide, depending on the organic waste digested and culture conditions (Rongwong et al. 2012). Figure 18.9 provides an overview of the anaerobic digestion process by which biomass is converted to biogas inside a conventional anaerobic digester.

Due to the large fraction of CO_2 in biogas, natural gas has a significantly higher energetic content which is one reason biogas has a difficult time competing economically. Biogas can be combusted directly for some applications like cooking. For more energy-intensive applications, removal of CO_2 is essential for biogas "enhancement" to concentrations comparable to natural gas; however, this is energetically costly (Wahid et al. 2019). Accordingly, methods to diminish CO_2 and enhance CH_4 in biogas are of interest, and various chemical, physical, and biological methods exist (Jourdin et al. 2016). Biologically, photosynthetic microbes have been successfully employed as an efficient means of scrubbing up to 99% of CO_2 from biogas while facilitating autotrophic growth by overcoming the CO_2 limitation inherent to air, which is only 0.04% CO_2 (Nagarajan et al. 2019; Pisciotta 2014). Algal removal of biogas carbon is beneficial because not only is CO_2 removed, but it

is converted into useful biomass including algal oil. Another biogas enhancement method delivers H_2 from a MEC to enable resident hydrogenotrophic methanogens to reduce additional CO_2. Using that approach, Xu et al. found the hydrogenotrophic methanogen *Methanobacterium petrolearium* contributed to the significant removal of CO2, producing biogas with roughly a 10% CO_2 content under mesophilic culture conditions (Xu et al. 2014). A dual-chamber, thermophilic reactor design enabled up to 98% methane biogas to be produced under laboratory conditions (Liu et al. 2017). Though H_2 may be used as an indirect electron carrier to support biogas enhancement, that is not required.

In 2009 Cheng and coworkers demonstrated that methane can be produced from the bioelectrochemical reduction of CO_2 in BESs through a phenomenon referred to as electromethanogenesis (Cheng et al. 2009). This methane can be produced either by direct reduction of CO_2 via cathode-associated methanogens or it may be mediated by H_2 through the activity of hydrogenotrophic methanogens (Cheng et al. 2009; Lohner et al. 2014; Siegert et al. 2015). Direct electromethanogenesis can commence at −400 mV vs. SHE (Beese-Vasbender et al. 2015). Direct electron transfer appears to be responsible for a minority of the CH_4 formed in some electromethanogenic reactor configurations (Zhao et al. 2016). Nevertheless, genetically modified *Methanococcus maripaludis* featuring deletion of hydrogenases still produced methane at the cathode, indicating the involvement of a direct, hydrogenase-independent pathway of electron uptake and CO_2 reduction to CH_4 (Lohner et al. 2014). Metagenomic microbial community analysis indicates that compared to conventional anaerobic digesters, MECs have significant increases in bacteria resulting in improved overall conversion of organic waste into VFAs that can subsequently be fed into CH_4 formation (Lee et al. 2017). Diverse microbes consume hydrogen as a source of high-energy electrons using enzymes such as bidirectional hydrogenases. Examples of microbes that consume hydrogen include hydrogen bacteria and certain cyanobacteria, while under the anaerobic conditions in a MEC, acetogens compete with a group of methanogens for H_2 substrate (Molenaar et al. 2017).

Pure cultures of methanogens tend to have lower current densities and methane production rates than mixed cultures (Logan et al. 2019). Genera of archaea detected in methanogenic BESs include *Methanobacterium*, *Methanococcus*, and *Methanobrevibacter* (Cheng et al. 2009; Lohner et al. 2014; Siegert et al. 2015). Since methane as a commodity is usually of lower value than hydrogen, inhibition of both types of methanogens can provide for increased ratios in favor of the more valuable H_2 (Nath et al. 2021). Hydrogen is also a clean-burning carbon-free fuel that can power fuel cells or conventional ICE vehicles and even certain jet engines (Baroutaji et al. 2019; White et al. 2006). Although electrohydrogenesis for the production of H_2 may be favorable over electromethanogenesis from an economic standpoint, this might not be practical nor desirable in some settings, for instance, bioelectrochemical boosting of anaerobic digesters in the agricultural or waste treatment sectors for the storage of renewable energy such as wind or solar (Noori et al. 2020; Pisciotta 2019). An electromethanogenic BES being used for wind energy storage as methane gas is shown in Fig. 18.10.

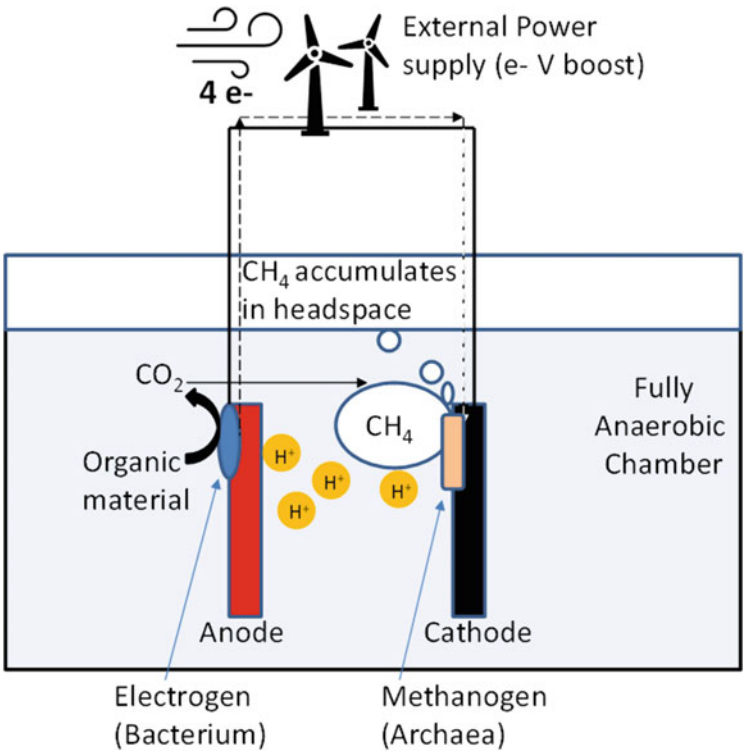

Fig. 18.10 Electromethanogenic BES. Electrogenic microbes on anode catabolize organics, generating CO_2 plus protons as electrons are donated to anode, building a voltage that flows as DC current to the cathode. Electrons exit the cathode and reduce CO_2 in the presence of protons generating end product CH_4. Input energy is supplied by external wind turbines for requisite voltage boost since electromethanogenesis is not spontaneous. Here CH_4 represents stored, renewable wind plus waste energy

Microbes present on waste collectively catabolize waste to form biogas. Bacteria do not directly produce methane but generate substrates consumed by two groups of methanogenic archaea. Acetoclastic methanogens, such as *Methanosarcina*, consume acetate produced by the acetogenic bacteria, converting the methyl group to methane (Ferry 1997). Acetoclastic methanogens are responsible for most of the methane formed since few other microbes can compete with them for acetate. The second group is hydrogenotrophic methanogens. They consume and oxidize hydrogen gas as electron donors and energy sources as they reduce carbon dioxide to methane according to the following formula (Sancho Navarro et al. 2016):

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \tag{18.2}$$

The hydrogenotrophic methanogens compete for substrate H_2 with acetogens, limiting the amount of methane produced via this route. Conversely, the product of

Fig. 18.11 Anaerobic digesters of the Back River wastewater treatment plant in Baltimore, MD. On-site renewable electricity generation of high-efficiency, gas turbine generators produces 3 MW continuously plus heat for these thermophilic digesters. This reduces the energy-related operating cost an average of $1.4 million annually, according to Baltimore City Dept. of Public Works. This industrial process can be enhanced using electromethanogenesis. (Photo by Mark Jonas)

the acetogens, acetate, serves as the substrate of the acetoclastic (i.e., acetotrophic) methanogens. Consequently, acetoclastic methanogens are responsible for the majority of methane formed in wastewater treatment anaerobic digesters (Guo et al. 2015). Figure 18.11 shows large industrial anaerobic digesters that help power the Back River industrial wastewater treatment plant in Baltimore, MD, with biogas combusted to generate renewable electricity. A variety of strategies have been used to boost methane production and aid the conversion of waste lipids into methane (Kurade et al. 2019). Algae and other phototrophs have been successfully employed as an efficient means of scrubbing up to 99% of CO_2 from biogas (Nagarajan et al. 2019; Pisciotta 2014). Algal enhancement of biogas is useful because not only is CO_2 removed but it is converted to lipid-rich algal biomass. Electromethanogenesis can similarly be applied to industrial anaerobic digesters to enhance biogas formation while storing renewable solar or wind power as methane gas.

18.3.4 Electrofermentation

Electricity can be applied to redirect existing microbial metabolic pathways toward the formation of desirable high-value products including biofuels, a process called electrofermentation (Chandrasekhar et al. 2015). Electrofermentation is a fairly new and promising subtopic within the expanding field of microbial bioelectrochemistry. Fermentative microorganisms normally produce diverse end products such as CO_2 and various alcohols, like ethanol. Such fermentation products are generated from the reduction of metabolic intermediates using electrons produced from substrate catabolism. In electrofermentation, also called electrically driven fermentation, controlled electrode potentials are used to modulate redox balance to shift cellular metabolism toward a more desirable end product (Chandrasekhar et al. 2015; Villano et al. 2017). Soluble mediators or electron shuttles, such as neutral red, may be used to facilitate electron transfer reactions (Paiano et al. 2019). In a methyl viologen-mediated electrofermentation by *Clostridium acetobutylicum*, butanol production was boosted 26%, while acetone was decreased (Kim and Kim 1988). Administration of external mediators or electron shuttles is not required for electrofermentation. Fermentation of glucose by axenically cultivated *Clostridium pasteurianum* was shifted in favor of elevated butanol production as a means of oxidizing NADH when grown in the presence of a cathode (Choi et al. 2014).

Interestingly, the mere presence of charged electrodes without electric current transfer (i.e., without cathode electron delivery) has been reported to both increase and accelerate ethanol production by *S. cerevisiae*, possibly by stimulating electron transfer rates within cells (Mathew et al. 2015). Future research of yeast-mediated electrofermentation might be developed for enhanced corn or cellulosic fermentations. Condensation of acetate and ethanol into butyric acid has been reported, suggesting an alternative mean by which this technique might be used to convert the lower-value, small molecules into higher-value, drop-in biofuels such as butanol (Paiano et al. 2019). The amount of 1,3-propanediol produced from glycerol by *Klebsiella* bacteria has recently been enhanced using electrofermentation (Kim et al. 2020). *C. pasteurianum* can also produce heightened 1,3-propanediol as a result of electrofermentation of glycerol (Choi et al. 2014). Since glycerol is a major waste produced in abundance by the biodiesel industry, electrofermentation could be useful for product valorization or value upcycling of microbial metabolites. Electrofermentation has yet to be broadly deployed at industrial levels for the production of biofuels or other products. Nevertheless, this method holds tremendous industrial potential.

18.4 Conclusions

Throughout society, electroactive microorganisms can play diverse industrial roles in the conversion of low-cost, available energy and wastes into valuable resources including energy storage molecules. Chief among these is bioelectricity production and electrosynthesis of fuels like green hydrogen and methane. Next-generation

drop-in biofuels, like electrosynthetically produced butanol, hold great promise as electrofuels. Electrically powered fixation of CO_2 and N_2 in certain bioelectrochemically active microbes, called electrotrophs, is an expanding area of microbiology with much basic sciences and applied industrial uses. Anaerobic digestion has been used for decades to treat wastes, and its use is increasing in many regions yet the basic process can be further enhanced through electromethanogenesis. Recently devised BESs, such as MFCs and MECs, can help treat wastes while generating DC electricity (MFCs) or storing renewable energy as clean-burning green hydrogen (MEC). Wastewater treatment plants could be transformed from energy consumers to clean electricity providers if modular MFCs can be improved and optimized for scale-up. In light of the ongoing climate crisis, the production of green H_2 using MECs appears particularly promising as a method for both treating wastes and storing renewable energy. Eventually, electrically driven fixation of atmospheric nitrogen into protein using electrodiazotrophic microbes could help to alleviate water and land use issues pertaining to the modern food industry. In the future, novel electrotrophic microorganisms may be applied for an expanded range of industrial applications such as the electrosynthesis and electrofermentation of high-value designer molecules, including pharmaceuticals, by genetically modified, electrically active microorganisms.

Conflict of Interest Statement No conflicts declared.

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Microbial-Assisted Systems for Lignin-Based Product Generation

19

Harit Jha and Ragini Arora

Abstract

Lignin is an abundant polyphenol found in the plant cell wall. In an enzyme-catalysed reaction, the monomeric monolignols p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol as phenylpropanoids form lignin. The composition of lignin differs across the plants with respect to the presence of monomers. It is difficult to degrade and act as a recalcitrant in the carbon cycle. Structural heterogeneity of lignin is a major hindrance in the bioconversion of specific by-products.

Basidiomycetes, white-rot fungi, brown-rot fungi, and certain aerobic bacteria can partially and totally degrade lignin with or without the use of mediators. Major enzymes involved in lignin degradation include manganese peroxidase, lignin peroxidase, versatile peroxidase, and laccase. The microbial-assisted systems with their enzymes can modify and biotransform lignin into a wide range of small molecular weight products. Lignin is an economical relatively non-toxic and renewable substrate for biotransformation processes. Lignin and its degradation products can be utilised for a variety of industrial applications including flavouring agents, polymers, biodegradable plastics, adhesives, fillers foam, insulators, etc. They have proven to have therapeutic benefits such as anticancer, anti-inflammatory, antioxidants, antibiotics, and antimicrobials agent.

Keywords

Lignin · Recalcitrant · Enzymes · Lignicolous fungi · Biotransformation · Industrial applications

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_19

Abbreviations

API	Atmospheric pressure ionisation
DMSO	Dimethyl sulphoxide
DyP	Dye-decolourising peroxidase
FTIR	Fourier transform infrared spectroscopy
FTMS	Full scan mass spectrometry
HBT	1-hydroxybenzotriazole
HRMS	High-resolution mass spectrometry
Lac	Laccase
LiP	Lignin peroxidase
LLE	Liquid-liquid extraction
MALDI	Matrix-assisted laser desorption/ionisation
MnP	Manganese peroxidase
NMR	Nuclear magnetic resonance
PA	Polyamide
PE	Polyester
PLA	Polylactic acid
PO	Polyol
PU	Polyurethane
SEC	Size exclusion chromatography
VP	Versatile peroxidase

19.1 Introduction

Lignin is a stable aromatic heteropolymer that makes up 10–35% of lignocellulosic biomass in terms of weight. It's a complex blend of high-molecular-weight insoluble aromatic polymers (Fig. 19.1). It is synthesised by free radical oxidative coupling among three monomers derived from the phenylpropanoid pathway (Gellerstedt et al. 2008). It makes up a rigid, impermeable structure resistant to microbial attack and oxidative stress and contains well-built cellulose microfibrils due to a number of alkyl−/aryl-ether interunit linkages. It is present in around 10–25% of the biomass by weight in the plant primary cell wall and makes up a rigid, impermeable structure resistant to microbial attack and oxidative stress and contains well-built cellulose microfibril (Duval and Lawoko 2014).

Lignin acts as a molecular bond, protecting and binding cellulose and hemicellulose. Lignin is a vascular plant tissue that provides mechanical protection to plant tissue and individual fibres, as well as physical strength and rigidity to the biomass cell structure. The amount of lignin in different lignocellulose biomasses varies.

Lignin is a possible feedstock for biodegradable polyhydroxyalkanoates, polyhydroxybutyrate, and polylactic acid (PLA) and non-biodegradable (polyurethane (PU), polyolefin (PL), polyester (PE), polyamide (PA), and polyol (PO))

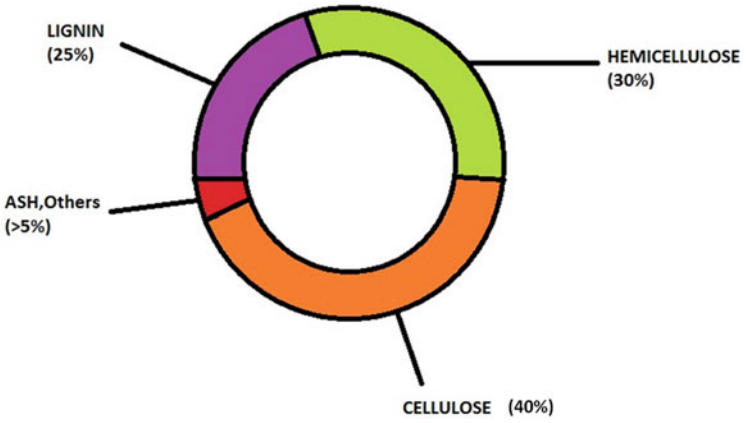

Fig. 19.1 Compositional distribution of lignocellulose as hemicellulose (30–35%), cellulose (40–45%), and lignin (25–30%)

bio-based polymers. Lignin is also used as a dispersing agent, concrete additive, gasoline, animal feed additive, vanillin processing, resins, and adhesives as well as an alternative fuel for power and heat generation (Capanema et al. 2005).

Lignin has emerged as a major structural compound in nature, especially in terrestrial plants. Lignin is one of the three main components of lignocellulose, the woody component of plants, along with cellulose and hemicellulose. It acts as a systemic stabiliser, a water pipeline inside plants, and a buffer against microbial decay in this situation.

19.2 Structure of Lignin

Lignin is an important part of plants that constitutes up to 30% of the biomass of plants (Ragauskas et al. 2014). As the second most abundant natural polymer on Earth after cellulose and the only aromatic green feedstock, it plays an important role in providing structural stability by strengthening plant cell walls, regulating fluid flow, and allowing pathogen resistance. Lignin serves as a matrix in nature, holding the plant together and protecting it from environmental conditions (Davis et al. 2016).

The same characteristics that make lignin valuable to plants often make it difficult to extract and refine into industrially useful materials. It's a three-dimensional long-chain, complex, amorphous substance and heterogeneous aromatic polymer of 3C phenylpropanes with a large molecular weight connected to 6C atom rings, methoxy clusters, and non-carbohydratic polyphenolic compounds connected by ether bonds.

Chemically, lignin consists mainly of three phenylpropanoid units: sinapyl alcohol (S), coniferyl alcohol (G), and p-coumaryl alcohol (H) monolignols, with alkyl-alkyl, aryl-aryl, and alkyl-aryl group linkages that specify the biological and physicochemical properties of the polymer lignin (Ponnusamy et al. 2019)

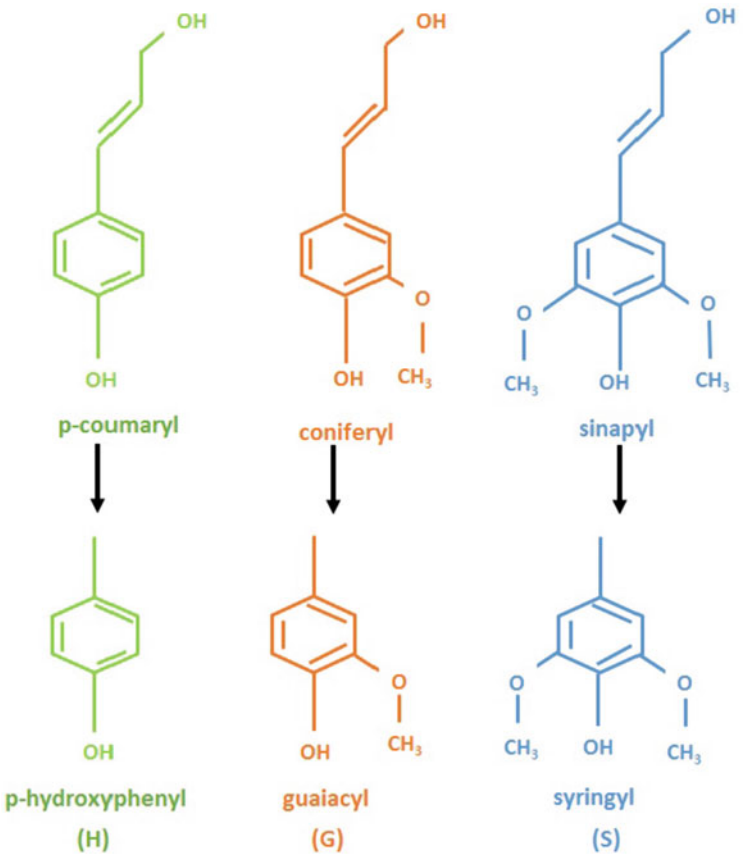

Fig. 19.2 Chemical structure of monolignols and their building blocks in lignin

(Fig. 19.2). The fundamental structure of phenylpropanoids binds these three units, but in their phenolic rings, they differ in the number of methoxy groups (Duval and Lawoko 2014). The G unit contains monomethoxy phenoxide, the S unit contains dimethoxy phenoxide, and the H unit contains a non-substituted phenoxide moiety.

The structure and composition of lignin are primarily determined by the biomass source. The lignin structure differs from material to material with distinctive monomer proportions of softwoods and hardwoods; such composition variability signifies that the existence of various lignin bonds differs from plant to plant.

Softwood contains the best-quality lignin, which is largely made up of coniferyl units (G-type lignin). Hardwood lignin, on the other hand, is composed almost entirely of coniferyl and sinapyl alcohol (G/S-type lignin), while grass, herbaceous, and angiosperm lignin also comprises p-coumaryl (G/S/H-type lignin) alcohol units.

The dominant linkage in lignin, β-aryl ether (β-O-4) bonds, will account for 50% of softwood lignin and 80% of hardwood lignin, respectively. Among other linkages, 5–5, β-5, 4-O-5, β-1, and β-β are resistant to most degradation processes.

Unlike the homopolymer cellulose [β-(1- > 4) linkages in D-glucopyranose units], the heteropolymer lignin has a-OH, CH3-O, R-COOH, and >C-O functional groups that specify lignin reactivity (Gellerstedt et al. 2008).

For the efficient use of renewable fuels, materials, or chemicals, structural analysis of lignin and recognition of the essence of the lignin linkages are needed. Lignin contains many C–C acid-resistant compounds. Lignin is largely degraded by an oxidative attack on C-C bonds and is only partly reduced to monomeric compounds by hydrolysis (Martrinez et al. 2005). Its transformation into desired products is hampered by biomass fractionation, depolymerisation, and component upgrades.

There are different approaches for disclosing the molecular specifics of the composition of lignin. The degradative approaches exposing the H/G/S structure of the lignin polymer are pyrolysis-gas chromatography-mass spectrometry (GC-MS), nitrobenzene oxidation, thioacidolysis and derivatisation with reductive cleavage. These techniques release just a fraction of the polymer for analysis. Only some H, G, and S units b-O-4[ether] bound to other units are released from the polymer and GC-MS quantified from the so-called polymer non-condensed fraction by thioacidolysis and derivatisation followed by reductive cleavage. NMR spectroscopy, especially two-dimensional (2D) 13C-1H correlated (HSQC, HMQC) spectroscopy, which combines the sensitivity of 1H NMR with the higher resolution of 13CNMR, remains the best method for revealing the frequencies of the various lignin units and the interunit bonding patterns (Capanema et al. 2005).

19.3 Major Systems Developed for Lignin Bioconversion

Lignin is constantly underutilised, with the majority of manufactured lignin, approximately 70,000,000 metric tonnes per annum, being used just as a combustible substance due to its high heat value. Furthermore, separating lignin from biomass can be a time-consuming and intensive process and often necessitates the use of harsh chemicals.

Since lignin is a sustainable and underutilised resource, comprehensive research has been conducted to improve conversion methods for transforming lignin into high-value products. The aromatic biopolymer's structural complexity and recalcitrance, which is bound together arbitrarily by tight CC and CO bonds, make lignin depolymerisation an exciting process.

Lignin contains a variety of linkages, which differ in form and ratio depending on the plant source. The β-O-4 linkage is the most common, accounting for roughly half of all linkages and hence the target of most depolymerisation strategies. The vast majority of catalytic processes tend to impact depolymerisation through scission of the CO linkage in the β-O-4 moiety since it is the most abundant.

Any efficient lignin depolymerisation method should yield and pick unique (preferably aromatic) compounds in adequate amounts to allow separation and eventual valorisation to specific products (Bhardwaj and Verma 2021; Bhardwaj et al. 2021; Kumar and Verma 2021a, b). The conversion of lignin to aromatic/

phenolic compounds of low molecular weight is a promising pathway for lignin to be used in high-value applications (Bhardwaj et al. 2020; Kumar and Verma 2020a, b). Precise control over the methods used to selectively depolymerise lignin is expected to propel this field even further into new realms of synthetic chemistry centred on using lignin as feedstocks.

Based on previous studies, there are various methods for degrading lignin; an overview of some of them is given below.

19.3.1 Pyrolysis

Pyrolysis is the process of heating biomass in the absence of oxygen. At comparatively high temperatures (up to 500 °C), biomass is converted to a liquid (bio-oil), gas, and solid (char) by fast pyrolysis (Fig. 19.3).

It's an anoxic thermal degradation mechanism in which the pyrolysis reactor is rapidly heated to the target temperature (400 to 600 °C), allowing the materials within to easily decompose into volatile compounds, accompanied by rapid condensation to a liquid state. Monomeric phenolic compounds, acetic acid, and large concentrations of char are the primary products of lignin pyrolysis.

Bio-oil is made up of chemicals that are structurally identical to the original molecules and accounts for up to 75% of the pyrolysis product. However, the pyrolytic lignin has fewer methoxyl groups than lignin derived from milled wood; this is most likely due to the demethoxylation of guaiacyl and syringyl moieties, which results in the formation of methanol. Carbohydrate-derived compounds have a higher water affinity than lignin-derived compounds in bio-oil. Water, regulated

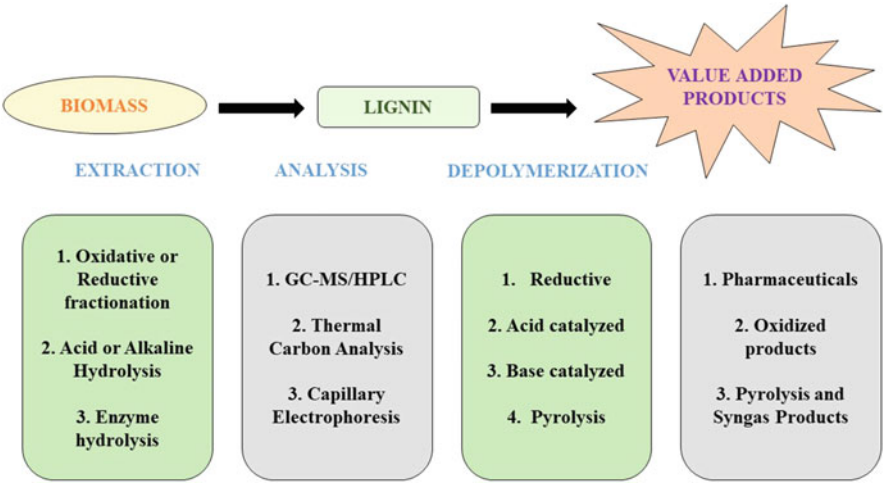

Fig. 19.3 Overview of the conversion of lignin into value-added products

deposition, or solvent extraction may all be used to separate the lignin component. Biomass pyrolysis also contains biochar, a solid that can be used as carbon sequestration and crop enhancement soil amendment.

As these conditions for reaction can be readily optimised to produce bio-oil of excellent consistency, fast pyrolysis of lignin is more efficient and viable than traditional thermochemical methods.

19.3.2 Oxidative Depolymerisation

Polyfunctional aromatic compounds formed by oxidative depolymerisation of lignin include aromatic acid, syringaldehyde, and vanillin. The oxidative cracking reaction involves the cleavage of β-O-4 bonds, C-C bonds, aromatic rings, or other linkages within the lignin (Cao et al. 2019).

Because of the abundance of hydroxyl groups in lignin, oxidative cracking could be an attractive option to explore. It uses an oxidant like metal oxides or hydrogen peroxide to cleave the lignin linkages. The major components of the oxidative breakdown of lignin are aromatic aldehydes and carboxylic acids (Qi et al. 2016a, b). The oxidative deconstruction of lignin produces vanillin as a primary component, with yields ranging from 5 to 15% of the lignin source (Xu et al. 2014).

For the production of chemicals (e.g. aromatic acids, aldehydes) where the aromatic moieties are entirely preserved as well as potential room for ring-opened organic acids, oxidative depolymerisation techniques can be relatively helpful. The hydroxyl groups are also attacked by these oxidants, which transform them into aldehyde, ketones, and carboxylic acids, among other things.

19.3.3 Reductive Depolymerisation

Reductive depolymerisation is an effective method for lignin conversion due to its high selectivity in lignin C-O and C-C bond cleavage (Van den Bosch et al. 2015). The products of this process have a heavy ratio of hydrogen to carbon (H/C), making them suitable for biofuel processing (Cao et al. 2019). Reductive lignin treatment can be followed back to early structural elucidation experiments in which lignin was treated with Cu Cr catalysts under relatively harsh reaction conditions (250–260 °C, 220–240 bar) in order to get aliphatic compounds (primarily 4-propylcyclohexanol M3), which had been isolated and characterised primarily boiling or melting points, as well as elemental analyses.

Catalysts that can selectively break CO bonds, resulting in depolymerisation, are required for reductive approaches. It has recently been demonstrated that utilising H-donating solvents, like formic acid, and other stabilising compounds as in alcohol, will cleave the β-O-4 linkage and use reductive pathways to stabilise the reactive species that results. Due to their cost-effectiveness and high activity, Ni-based catalysts are commonly used in catalysis in industry. Over Ni-based catalysts,

selective cleavage of β-O-4 in the hydrodeoxygenation process can be supported for reductive conversion of lignin.

19.3.4 Base-Catalysed Depolymerisation

When lignins are exposed to high-degree temperatures and pressures in the presence of a base in an aqueous or organic solution, they may make low-molecular-weight compounds (Chandra and Bharagava 2013). The base-catalysed depolymerisation of three related organosolv lignins (acetosolv, acetosolv/formosolv, and formosolv) was investigated, which revealed that base-catalysed depolymerisation of acetosolv and acetosolv/formosolv lignins yielded a greater yield of desired oil product. Low-cost inorganic bases, such as alkali metal hydroxides, are widely used as catalysts for base-catalysed lignin depolymerisation. During the base-catalysed delignification step, β-aryl ether bonds are cleaved to form quinone methide intermediates from phenolate groups (Lavoie et al. 2011; Dabral et al. 2018).

When compared to a control with no alkaline, the application of sodium hydroxide will boost the yield of depolymerised lignin by more than fourfold. Because of its low cost, NaOH was widely used in industry as a catalyst for lignin depolymerisation. Instead of NaOH, other bases such as KOH, CsOH, $Ca(OH)_2$, and others have been investigated. It's worth noting that Evans et al. discovered that using strong bases such as KOH and NaOH would transform and produce more depolymerised products than using weak bases such as $Ca(OH)_2$ and LiOH (Evans et al. 1999).

Furthermore, the strong base will suppress char formation while maintaining the reactivity of phenolic compounds during depolymerisation. These organic solvents, however, may also form a compact structure with the lignin molecule (Jasiukaitytė et al. 2010). Therefore, the carboxylic acids formed during depolymerisation can lower the pH of the reaction mixture further, resulting in repolymerisation. On the other hand, demonstrated that lignin depolymerisation in water cannot be catalysed by a low concentration of base catalyst, with almost no depolymerised products observed (Knill and Kennedy 2003).

As a result, instead of water, most studies use organic solvents such as ethanol, polyethylene glycerol, and isopropanol (Mahmood et al. 2015; Jin et al. 2011; Wang and Rinaldi 2013; Paola and Roberto 2014). Thus, during base-catalysed depolymerisation, repolymerisation is observed which may reduce the efficiency of depolymerisation and increase the amount of residual lignin.

19.3.5 Acid-Catalysed Depolymerisation

Hägglund and Björkman in 1924 conducted the first acid-catalysed lignin hydrolysis reaction, distilling lignin with 12% hydrochloric acid and attempting to produce thiobarbituric acid, phloroglucinol, and barbituric acid. Several acids, including

mineral and Lewis acids, zeolites, ionic liquids with Bronsted acidic functionalities, and organic acids, have recently been studied for their ability to depolymerise lignin.

Since the α- and β-aryl ether linkage has lower activation energy than the β-aryl ether linkage for acid-catalysed hydrolysis, the former linkage undergoes faster depolymerisation than the latter. At temperatures up to 150 °C, acid-catalysed cleavage of the β-O-4 linkage in lignin-based model compounds revealed two major pathways: path A, which leads to the formation of Hibbert's ketones (C3 fragments), and path B, which includes the loss of formaldehyde molecule, followed by the formation of ethyl benzene derivatives (C2 fragments), as well as repolymerisation reactions (Sturgeon et al. 2014; Yokoyama 2015).

In the rate-determining process, β-O-4 linkages are cleaved of acid-catalysed dehydration, giving an enol aryl ether intermediate that hydrolyses easily to guaiacol and ketocarbinol. By allylic rearrangement, these compounds eventually convert into Hibbert's ketones. Hibbert's ketones (phenolic C3 fragments) are the principal monomeric products of acidolysis of lignin, whereas C2 fragments are obtained insignificantly less due to the presence of unstable C2 aldehyde in the repolymerisation reaction, which often results in the development of high-molecular-weight polymers (Xu et al. 2014; Roberts et al. 2011).

19.4 Microbial Degradation of Lignin

The plentiful biomass of lignocellulose offers an alternative outlet for the manufacture of green fuels and chemical compounds. As a consequence, its depletion is essential to the global carbon cycle's activity. The most intractable portion of the biopolymers are lignins, and only a small number of microbial species are capable of degrading lignins because of their multifaceted nature (Sen et al. 2014).

Different chemical catalyst-assisted therapies, such as supercritical fluids and ionic liquids assisted and metal, acid and base catalysed, assisted, and thermochemical treatments, such as gasification, hydrogenolysis, pyrolysis, chemical oxidation, and supercritical hydrolysis, have demonstrated enormous potential for highly efficient lignin depolymerisation. However, they require extreme reaction conditions, including elevated pressure and temperature. In addition, these systems typically have risk factors in the environment that can cause environmental harm and require a large amount of energy (Pandey and Klm 2011).

The enzyme-based depolymerisation of lignin has also been suggested as an alternative approach. Since the enzymes or microbes used in biological therapies are typically natural and non-toxic to the ecosystem, biocatalyst is commonly thought to be an environmentally friendly trigger (Bugg et al. 2020).

Furthermore, since certain enzymes can catalyse the same reaction directly, using a biocatalyst can boost the reaction's selectivity and avoid unwanted side reactions like repolymerisation. Understanding the microbial reactions that facilitate the release of this carbon will help us better understand carbon cycling and how it contributes to climate change. The degradation of lignin in nature, with a few cases of bacterial ligninolytic involvement, is primarily carried by fungal enzymes. This

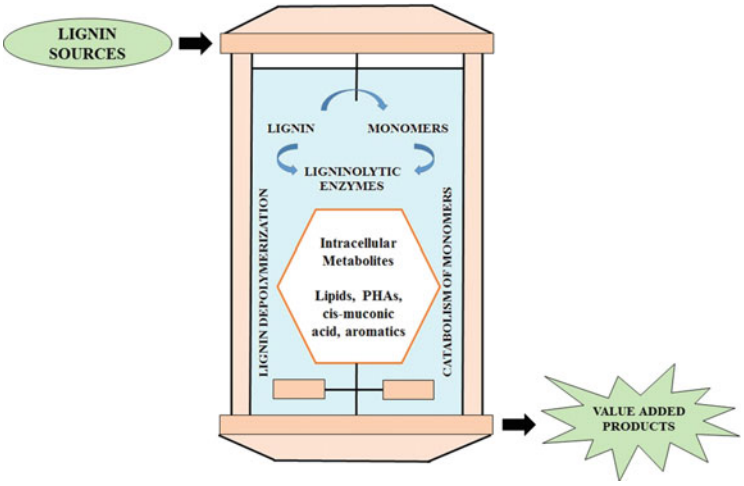

Fig. 19.4 A visual representation of the consolidated lignin bioprocessing concept

primary oxidation produces a number of low-molecular-weight aromatics, which bacteria use as carbon and energy sources.

If the biocatalytic breakdown of lignin can be harnessed and monitored, it could become a renewable source of phenolic and aromatic substances, fine chemicals, and materials for the food and flavour industries (Palazzolo and Kurina-Sanz 2016). A schematic representation of the proposed procedure is presented in Fig. 19.4.

19.4.1 Ligninolytic Microorganisms

The most prevalent phenolic polymer in nature is lignin, and many species have evolved efficient metabolic systems and methods for degrading and converting lignin to aromatic compounds, which are then converted to energy through multiple pathways.

Biological lignin processing has arisen as a potentially lucrative method of manufacturing lignin-derived products. The ability to use green carbon sources as feedstock for the manufacturing of various intermediates to support cellular growth and the ability to generate target molecules with an accuracy that surpasses synthetic chemistry are two benefits of biological processing (Chubukov et al. 2016; Liu et al. 2013; Wan and Li 2012; Asina et al. 2017).

The ability of microorganisms to actively produce desired chemicals with special properties could be used to generate new materials for particular biofuels or chemicals. Several enzymes and related small-molecule co-factors have been shown to degrade lignin by fungi and bacteria in vitro and/or in vivo tests.

19.4.1.1 Lignin Degradation by Fungi

Microorganisms have two kinds of extracellular enzyme systems: one that induces hydrolysis and is responsible for cellulose and hemicellulose degradation and another that depolymerises lignin (Li and Zheng 2019; Agrawal and Verma 2020; Agrawal et al. 2019). Enzyme combustion is the microbial degradation of lignin into lesser fractions (Beckham et al. 2016). For depolymerisation and oxidation of lignin, the fungus is one of the most studied microbes. Currently, lignin is degraded by fungal organisms using their powerful lignin-degrading enzyme systems, which include phenol oxidases as well as quinone reductases, aryl alcohol oxidases, xylanase, glyoxal oxidases, endoglucanase, laccase, and other enzymes.

The stability of fungi in practical therapy is not attractive when exposed to such environmental factors as higher pH (>7) or anaerobic conditions. White-rot fungi are the most effective microbes in causing natural lignin in wood to deteriorate. Its high ability to dissolve lignin could be linked to the fact that rot fungi such as *Phanerochaete chrysosporium* can produce a variety of extracellular oxidases, including lignin manganese, laccases, and phenol oxidases (Brunow 2010; Bugg et al. 2011; Singh et al. 2013; Majumdar et al. 2014) (Tables 19.1 and 19.2).

Table 19.1 Previous research on fungal degradation of natural lignin in different biomass sources is summarised in this report

Fungal strain	Substrate	Reference
Fusarium proliferatum	Polymeric organosolv lignin	Perestelo et al. (1997)
Phanerochaete chrysosporium	Kraft lignin	Ulmer et al. (1983)
Ceriporiopsis subvermispora	^{14}C-labeled lignin	Ferraz et al. (2003)
Cladosporium Bio-1	Alkali lignin	Jin (2012)
P. chrysogenum	Kraft pine lignin	Rodriguez et al. (1994)
T. versicolor G20	Bamboo culms	Zhang et al. (2007)
L. betulinus	Wheat straw	Knežević et al. (2013)
F. moniliforme 821	Rice straw	Chang et al. (2012)
Ceriporiopsis subvermispora	*Eucalyptus grandis* wood	Tanaka et al. (2009)
Hymenoscyphus ericae	Alkaline lignin	Haselwandter et al. (1990)
P. ostreatus	Wheat straw	Knežević et al. (2013)
E. taxodii 2538	Bamboo culms	Zhang et al. (2007)
C. subvermispora	Japanese beech and cedar wood	Tanaka et al. (2009)
Galactomyces geotrichum	Rye straw	Varnaitė and Raudonienė (2005)
Aspergillus sp. F-3	Alkali lignin	Yang et al. (2011)
B. pumilus	Lignosulphonate	Arun and Eyini (2011)
Exidiopsis sublivida TUFC20068	Bamboo culms	Suhara et al. (2012)
Fusarium sp. 89	Rice straw	Chang et al. (2012)
Resinicium friabile TUFC20062	Bamboo culms	Suhara et al. (2012)
B. pumilus	Lignosulphonate	Arun and Eyini (2011)

Table 19.2 Bacterial degradation of natural lignin in different biomass sources

Bacterial strain	Substrate	Reference
Aeromonas sp.	Kraft lignin	Deschamps et al. (1980)
Acetoanaerobium sp. WJDL-Y2	Kraft lignin	Duan et al. (2016)
Serratia marcescens	Kraft indulin	Manangeeswaran et al. (2007)
Pandoraea sp. B-6	Kraft lignin	Shi et al. (2013)
Bacillus sp. EU978470	Alkali lignin	Abd-elsalam and El-Hanafy (2009)
S. viridosporus	Kraft indulin	Giroux et al. (1988)
Citrobacter freundii	Kraft lignin	Chandra and Bharagava (2013)
Azotobacter sp. HM121	Softwood kraft lignin	Morii et al. (1995)
Streptomyces sp. strains F-6	Alkali lignin	Yang et al. (2012)
S. badius	Lignin	Giroux et al. (1988)
Comamonas sp. B-9	Kraft lignin	Chai et al. (2014)
S. viridosporus T7A	Grass	Antai and Crawford (1981)
Bacillus sp. CS-1	Rice straw	Chang et al. (2014)
S. setonii 75Vi2	Maple	Antai and Crawford (1981)
I. lacteus	Corn stover	Song et al. (2013)
Lactic acid bacteria	Rice straw	Chang et al. (2014)
Microbial consortium XDC-2	Corn stalk	Hui et al. (2013)
S. viridosporuis	Corn stover	Pometto III and Crawford (1986)
Pleurotus ostreatus strain G5	Alkali lignin	Yang et al. (2012)
Bacillus sp. ITRC S6, S7, and S8	Kraft lignin	Chandra et al. (2007)

While lignin breakdown is inefficient in terms of energy, white-red basidiomycetes fungi have been discovered to completely break down lignin into CO_2 using ligninolytic enzymes, which require a lot of peroxides and laccases, whereas brown-red fungi could only change the structure of lignin significantly. Lignin peroxidase degrades nearly 90% of non-phenolic lignin polymer units, while manganese peroxidase converts Mn^{2+} to Mn^{3+}, allowing both phenolic and non-phenolic units to oxidise (Wong et al. 2009; Leonowicz et al. 1999; Salvachua et al. 2015; Picart et al. 2017; Kuatsjah et al. 2017).

Extracellular laccase and distinct peroxidase are the primary mechanisms by which fungi degrade lignin. Although fungi outperform bacteria in lignin degradation due to a powerful extracellular enzyme that is similar to other biologic lignin depolymerisation, the enzyme's efficiency is much less than chemical catalysis.

19.4.1.2 Lignin Degradation by Bacteria

More recently, bacterial lignin degradation has piqued scientific interest in fungi-based approaches, owing to the ease with which bacteria can be handled and genes manipulated. In addition to these characteristics, bacteria, unlike fungi, can easily adapt to a wide range of pH and thermal stability and provide a forum for a wider range of metabolic activities (Yang et al. 2017; Suman et al. 2016; Dwivedi et al. 2011; Chen and Wan 2017). The β-ketoadipate pathway degrades bacterial lignin, converting it into lipids and polyhydroxyalkanoates. Bacteria and fungi use

analogous enzymatic mechanisms of lignin depolymerisation; Dyp B, Dyp2, laccase, CopA, and etherase are among the bacterial enzymes capable of breaking lignin into smaller fractions (Fuchs et al. 2011) (Tables 19.1 and 19.2).

Several bacterial strains found in the intestines of wood-burning termites and beetles have been shown to effectively break down lignin. These bacteria strains were divided into three groups: *Proteobacteria* and *α-Proteobacteria* are actinomycetes, respectively (Yang et al. 2017; Tian et al. 2014).

19.5 Enzymes Involved in Lignin Degradation and Biotransformation

Lignin can be efficiently degraded using enzymes isolated from fungi or bacteria in several in vitro experiments for lignin depolymerisation or conversion studies.

The majority of in vitro studies using a single enzyme to cleave a lignin model molecule as a substrate. One of the biggest difficulties in deconstructing lignin, according to chemical processes of lignin degradation, is catalyst selectivity.

A variety of ligninolytic enzymes are required for biological lignin valorisation. A subset of these enzymes can generate aromatic radicals, which can then be used to cleave a variety of linkages through non-enzymatic reactions (Guillén et al. 2005; Xie et al. 2015; Sun et al. 2017; Fernandez-Fernandez et al. 2013) (Table 19.3).

Manganese-dependent peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP), and dye-decolourising peroxidase (DyP) are the four primary types of ligninolytic peroxidases (Fig. 19.5).

19.5.1 Lignin Peroxidase (EC 1.11.1.14)

LiP was discovered in *Phanerochaete chrysosporium* and has the ability to oxidise compounds with a high redox potential, such as mildly active non-phenolic aromatics, which make up to 90% of the lignin polymer.

It's a heme-containing glycoprotein with a molecular weight of 38 to 43 kDa and a heme group in the active region (Pérez et al. 2002). For the non-phenolic and phenolic compounds to be initiated and catalysed, hydrogen peroxide is required (Datta et al. 2017). To complete the catalytic cycle, veratryl alcohol was also needed as an electron donor and cofactor (Wong 2009) (Fig. 19.5).

LiP is also considered as the most powerful peroxidase when compared to other peroxidases because of its high redox potential, which enables LiP to oxidise substrates that other peroxidases cannot (Sigoillot et al. 2012). Due to its high efficiency in extracting lignin, LiP is also used for delignification in addition to lignin depolymerisation (Zhang et al. 2015; Vandana et al. 2018).

Table 19.3 Enzymes with ligninolytic activity

Organism	Enzyme	Substrate	Reference
Serpula lacrymans	LiP	MWL of pine	Ahmad et al. (2010)
Thermobifida fusca	DyP	VGE	Rahmanpour et al. (2016)
Penicillium citrinum	LiP	Veratryl alcohol	Kumari et al. (2002)
Dichomitus squalens	β-Etherase	β-O-4 lignin compound	Marinovic et al. (2018)
Escherichia coli	Laccase	PAH	Grass and Rensing (2001)
Pseudomonas putida	Laccase, MnP, LiP	Kraft lignin	Ahmad et al. (2010)
Lepista nuda	MnP, laccase	MWL of pine, wheat	Ahmad et al. (2010)
Anabaena sp. strain PCC7120	DyP2	Syringaldehyde	Ogola et al. (2009)
Aspergillus terreus	LiP	Veratryl alcohol	Kumari et al. (2002)

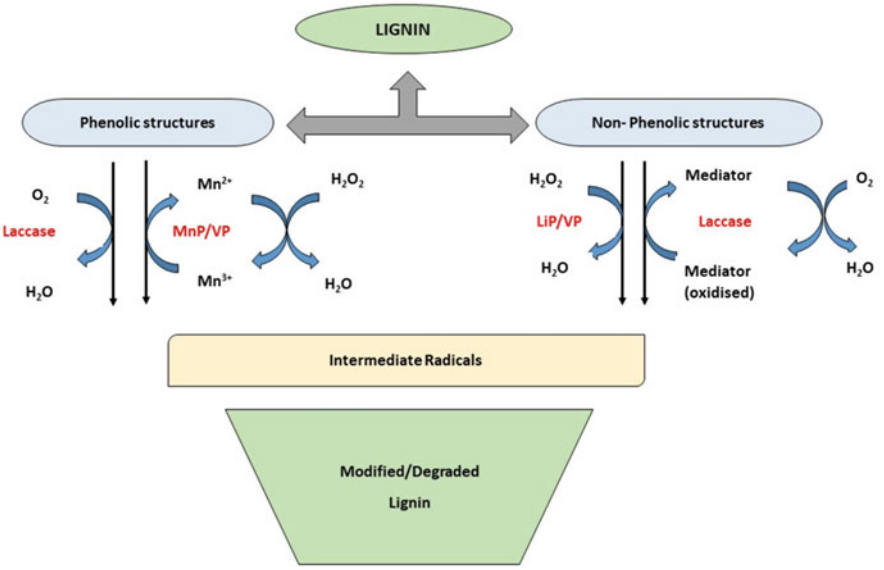

Fig. 19.5 Lignin-degrading enzymes catalyse the reaction. *MnP* manganese peroxidase, *VP* versatile peroxidase, *LiP* lignin peroxidase and laccase

19.5.2 Manganese Peroxidase (EC 1.11.1.13)

MnP, unlike LiP, cannot oxidise non-phenolics and therefore requires Mn2+ ions to work. In contrast, MnP will oxidise phenolic model lignin compounds. MnP has been shown to oxidise non-phenolic lignin-related materials, allowing the oxidising

ability of MnP to be transferred to Mn3+ and then diffused through the lignified cell wall to attack lignin from the inside.

MnP (manganese-dependent peroxidase) is a glycosylated protein that begins the catalytic cycle with hydrogen peroxide as an oxidant. MnP then uses Mn2+ as a reducing substrate to produce Mn3+. Mn3+ is a strong oxidant that diffuses from the enzyme and starts oxidising the lignin and phenolic compounds. As a consequence, Mn3+ can transform lignin phenolic compounds to phenoxy radicals, which can induce lignin depolymerisation (Hofrichter 2002). MnP, like laccase, plays an important role in the initial depolymerisation of lignin (Guillén et al. 2005) (Fig. 19.5).

In vitro tests also showed that the inclusion of MnP in the system would increase the depolymerisation process' effectiveness (Jayasinghe et al. 2011; Hettiaratchi et al. 2014).

Normally, MnP can only oxidise phenolic compounds; however, in the presence of additional Mn^{2+}, MnP can also oxidise non-phenolic lignin model compounds; previous research also showed that a high level of Mn^{2+} would increase MnP's activity to degrade lignin in solid (Rothschild et al. 1999; Kirk and Cullen 1998). Repolymerisation is also observed when LiP and MnP are used to depolymerise the synthetic lignin polymer (Hammel et al. 1993; Wariishi et al. 1991).

19.5.3 Versatile Peroxidase (EC 1.11.1.16)

The most recently discovered lignin oxidoreductase, versatile peroxidase, combines the characteristics of the LiP and MnP enzymes. Both non-phenolic and phenolic compounds can be oxidised by VP. For lignin depolymerisation, a variety of peroxidases can be used. *Bjerkandera* and *Pleurotus* fungi contain versatile peroxidase (VP), which shares certain catalytic properties with MnP and LiP (Hofrichter 2002; Moreira et al. 2007).

The enzyme VP has two functions. It can oxidise Mn^{2+}, such as MnP, as well as a variety of subtracts with high or low redox potentials, such as LiP (Ruiz-Dueñas et al. 2008; Camarero et al. 1999). Unlike MnP, however, VP can oxidise Mn^{2+} on its own (Perez-Boada et al. 2005). The bifunctionality of VP is also explained by protein crystal structural analyses (Perez-Boada et al. 2005; Sáez-Jiménez et al. 2015; Zeng et al. 2017), and VP has drawn many research interests as a result of its bifunctionality. In addition to lignin depolymerisation, VP may be used to delignify biomass and decolourise agricultural waste (Baratto et al. 2015; Kong et al. 2017).

19.5.4 Dye-Decolourising Peroxidase (EC 1.11.1.19)

In 1999, the first DyP was isolated from *Bjerkandera adusta* (de Gonzalo et al. 2016; van Bloois et al. 2010). Despite the fact that DyP has a different sequence and structure than other peroxidases, they have comparable catalytic properties and

mechanisms for oxidising substrates by using hydrogen peroxide and a mediator (Liers et al. 2014; Sugano et al. 2007; Brown et al. 2012).

DyPs can be divided into four groups based on their sequence features (Fawal et al. 2012). Types A, B, and C are widely found in bacteria, while type D is produced primarily by fungi (Abdel-Hamid et al. 2013). Both peroxidase activity and a trait are present in these four classes of DyP. Despite the fact that Mn2+ is a required mediator for type B DyP oxidation, certain type A DyPs lack Mn^{2+} oxidation activity and may oxidise substrates through alternative pathways (Singh et al. 2013; Rahmanpour et al. 2016).

It's worth noting that a new DyP was recently discovered that can oxidise the substrate with air oxygen and in absence of H_2O_2 (Avram et al. 2018). DyP from *Pseudomonas putida* MET94 was also subjected to a mutagenesis study in order to increase DyP performance in industrial applications through direct evolution (Brissos et al. 2017).

19.5.5 Laccases (EC 1.10.3.2)

Laccases are another enzyme that helps in the lignin breakdown process. Laccases have been shown to oxidise phenolic compounds found in lignin and convert molecular oxygen to water. They also catalyse the production of phenoxyl radicals from phenolic compounds, resulting in C-hydroxyl ketone oxidation, alkyl-aryl cleavage, demethoxylation, and C-C cleavage. Laccases are bulky and contain non-phenolic subunits that resist direct lignin polymer action.

Laccase is a blue-copper phenoloxidase that can oxidise phenolic compounds by accepting oxygen as an electron acceptor. The unstable phenoxyl free radical could be formed from oxidised phenolic compounds, which could lead to polymer cleavage (Gianfreda et al. 1999). Although laccase's activity is restricted to phenolic compounds, it can also work with the mediator to degrade non-phenolic compounds (Bourbonnais and Paice 1999).

Some of the mediators are small molecules that can transfer electrons, such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT) (Hilgers et al. 2018; Camarero et al. 2004). With the aid of the mediator, laccase can degrade approximately 80–90% of the lignin structure (Camarero et al. 2004). Laccase can also be used in other therapies besides depolymerisation, such as lignin-like chemical conversion and delignification (Lim et al. 2018; Li et al. 2018; Chen et al. 2012).

19.6 Analysis of Lignin Degradation Products

Cellulose, hemicellulose, and lignin are the three main constituents of lignocellulosic biomass. Ethanol has historically been manufactured using cellulose and hemicellulose. Despite the fact that lignin is produced in large amounts every year, largely as a by-product of the ethanol and paper industries, it has limited applications.

Despite the fact that lignin has been identified as a possible source of value-added materials, it is still largely used as a feedstock and fuel for the production of heat and electricity. Lignin and its degradation products are usually analysed chemically using a variety of methods, including spectroscopic, isolation, and thermal protocols.

Spectroscopic techniques such as Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) spectroscopy, and mass spectroscopy (MS) reveal information about lignin as a whole molecule, disclosing aromatic units and interunit linkages, as well as providing information on distinct functionalities. SEC (size exclusion chromatography) is frequently used to determine the molecular weight (MW) of lignin and the mass distribution of its degradation products.

19.6.1 Capillary Electrophoresis

The structural diversity of plant-derived phenolic compounds is taken into account when developing a suitable method for their analysis. For the analysis of compounds obtained from biomass hydrolysis, high-performance liquid chromatography (HPLC), gas chromatography (GC) alone and in combination with mass spectrometry (GC-MS), thin-layer chromatography (TLC), thin-layer electrophoresis (TLE), and isotachophoresis (ITP) (Pfeifer et al. 1984) are well-established approaches.

Capillary electrophoresis (CE) has emerged as a viable technique for separating charged and uncharged species over the last decade. It allows for the fast quantitative and automated analysis of complex mixtures with high precision and quick turnaround times. Sugars, inorganic ions, and phenolic compounds can all be determined with a single separation column using indirect UV detection methods and derivatisation procedures.

19.6.2 GC-MS/HPLC

GC-MS, GPC, HPLC, and isotachophoresis (ITP) (Pfeifer et al. 1984) can all be used to explore aromatic compounds obtained from lignin (Lupoi et al. 2015). Many lignin degradation products can be separated and identified using the GC system in particular. However, in everyday use, this technique has the drawback of requiring a time-consuming derivatisation step. HPLC gradient analysis with reversed stages, on the other hand, can be performed without derivatisation. GC-MS was used to reduce uncertainties caused by differences in retention time and possible peak overlaps (Pecina et al. 1986).

19.6.3 MALDI Mass Spectrometry

To characterise the molecular compositions, various analytical techniques were used (Bozell et al. 2011; Hanson et al. 2010; Hasegawa et al. 2011). Lignins' intrinsic complex structural motifs and the addition of extra heteroatoms during

biogeochemical cycles and chemical degradation processes (Fox et al. 1990; Tu et al. 2014) as well as the material's variable relative abundance make it difficult and tedious to decipher its structural composition and variation.

HRMS (high-resolution mass spectrometry) is a new instrumental approach to lignin analysis that works well. From very small amounts of a single lignin sample, full scan mass spectrometry (FTMS) (Qi and O'Connor 2014) can detect exact masses as well as elemental formulae for thousands of compounds (Qi et al. 2016a, b). In addition, subsequent tandem mass spectrometry (MS/MS) studies reveal more information about the internal structures (Morreel et al. 2010; Jarrell et al. 2014; Owen et al. 2012). In the analysis of complex mixtures, atmospheric pressure ionisation (API) approaches are probably the most widely used ionisation sources (Hughey et al. 2000; Hertkorn et al. 2008; Cho et al. 2012; Barrow et al. 2010; Headley et al. 2014; Headley et al. 2015; Qi et al. 2016a, b). API is not the only method for analysing complex mixtures; matrix-assisted laser desorption/ionisation (MALDI) has also been used as an ion source (Kew et al. 2018; Blackburn et al. 2017).

MALDI is a pulsed ionisation method that is ideal for millisecond shotgun experiments. Second, since lignins are aromatic compounds, they can be used as MALDI matrices to absorb light. There is limited data available in literature on the MALDI-MS analysis of lignin (Kosyakov et al. 2014, 2018; Albishi et al. 2019; Bowman et al. 2019).

19.6.4 Thermal Carbon Analysis

Thermal techniques such as thermogravimetric analysis (TGA) and pyrolysis (Py)-GC-MS (Lupoi et al. 2015) are widely used to treat lignin recalcitrance and limited solubility in common solvents.

They can be used to describe a sample as a whole (e.g. TGA) or to address individual constituents (e.g. Py-GC-MS) (Nunes et al. 2010). To evaluate reaction processes that determine the thermal stability of isolated lignin, TGA is commonly used in combination with differential scanning calorimetry (DSC) or FTIR (Ke et al. 2011).

Specific compounds or lignin pyrolytic breakdown products and degradation products may be precisely identified using Py-GC-MS (Gutierrez et al. 2006).

19.6.5 HPLC/MSn

HPLC/MSn has recently been demonstrated to be a powerful method for analysing complex mixtures made up of specific biopolymers. MSn (multi-stage tandem mass spectrometry) is a versatile instrument for specifically analysing multiple mixtures.

Mass spectrometry can offer high-resolution information on the molecular weights and elemental compositions of uncertain elements in complex mixtures. MSn provides structural details for individual components of complex mixtures at

the molecular level. However, this tool has not yet been used to describe lignin degradation materials.

The use of HPLC in combination with negative-ion-mode high-resolution MS3 study for the characterisation of lignin degradation products has been shown in the literature. This method was combined with high-resolution MSn to provide structural data for components of a true lignin degradation mixture.

19.7 Applications of Lignin Degradation Products

The expanding world population drives up demand for fuels and biochemicals, causing a slew of cultural issues such as energy security and environmental concerns (Schutyser et al. 2018; Sun et al. 2018). Overreliance on fossil fuels has raised concerns about climate change and an imminent energy crisis, necessitating studies into green and sustainable energy alternatives. The investigation of effective methods for converting lignin into useful goods piques the interest of a growing scientific community.

Lignocellulose is a readily available, carbon-neutral, and inedible bioresource that could be replenished in large quantities per year on a global scale. It is the most abundant and stable organic carbon source on the planet, making it the best choice for potential sustainable biorefineries (Cai et al. 2013).

In recent decades, lignin has evolved from a waste product used as a low-grade fuel and animal feed to a precious product used in polymers, adhesives, and other applications (Bajwa et al. 2019; Graglia et al. 2015; Sahoo et al. 2011; Seydibeyoglu 2012). Lignin valorisation has recently piqued the interest of other industries, such as the medical and electrochemical energy materials sectors (Table 19.4).

Cost-effective valorisation methods must be implemented to ensure the long-term viability and vitality of biorefineries. Through valorising their by-product lignin, the development of value-added lignin-derived co-products would help to improve the profitability of second-generation biorefineries and the paper industry (Kumar et al. 2020a, 2020b) (Fig. 19.6).

Despite the fact that several experiments have been performed to convert lignin into usable materials, owing to low yields and poor final product efficiency, only a handful of these attempts are commercially feasible. The difficulty and recalcitrance of lignin's composition, as well as the high reactivity of lignin degraded fractions, which are vulnerable to condensation reactions, cause difficulties in lignin valorisation. The effective conversion of lignin to desired products must overcome many issues associated with biomass fractionation, such as lignin and derivative characterisation, lignin depolymerisation, and product upgrading. Characterisation of native lignin is complicated due to its variability and inconstancy.

Table 19.4 Applications of lignin-derived products

Microorganism	Substrate	Product	Reference
Pseudomonas sp. B13	Benzoate	cis,cis-Muconate	Schmidt and Knackmuss (1984)
Pandoraea sp. ISTKB	Kraft lignin	PHA	Kumar et al. (2017)
Cupriavidus basilensis	Kraft lignin	PHA	Shi et al. (2017)
Pseudomonas putida KT2440	Ferulic acid	Vanillin	Graf and Altenbuchner (2014)
Rhodococcus jostii RHA045	Wheat straw cellulose	Vanillin	Sainsbury et al. (2013)
Rhodococcus opacus DSM1069	Kraft lignin	Lipid	Wei et al. (2015)
Streptomyces setonii	Ferulate	Vanillin	Muheim and Lerch (1999)
Corynebacterium glutamicum MA-1	Catechol	cis,cis-Muconate	Becker and Kuhl (2018)
Arthrobacter sp. T8626	Benzoate	cis,cis-Muconate	Mizuno et al. (1988)
Pseudomonas putida BM014	Benzoate	cis,cis-Muconate	Choi and Lee (1997)

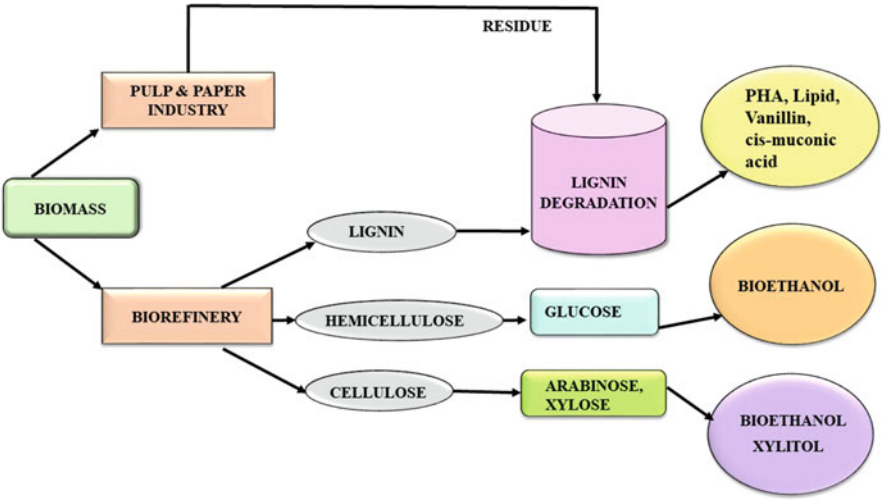

Fig. 19.6 Sources of lignin and pathways for producing valuable products from lignocellulosic biomass

19.7.1 Pharmaceuticals

Despite the fact that lignin-derived products are becoming more common in biomedical engineering, the majority of research is still in the proof-of-concept stage. Lignin-derived hydrogels and lignin-based nanotubes are widely used in tissue engineering and DNA distribution. The most popular way to use lignin is to depolymerise it and extract derivatives from which biologically active compounds can be synthesised.

Gil-Chávez et al. (2019) found that these biologically active substances have the ability to cure herpes simplex virus, influenza virus, and other viruses. Since the hydroxyl functional groups in lignins' phenolic rings neutralise free radicals and shield molecules from oxidation, their antioxidant property is highly valued when synthesising prescription drugs. Lignin-derived components and products, such as polyphenols, may aid in the management of various diseases.

19.7.2 Pyrolysis and Syngas Products

Solid biochar, bio-oils, or gases can be retrieved from lignin by heat treatment process called pyrolysis (Figueiredo et al. 2018). Biomass can be subject to different temperature and time regimes to obtain varying products like char, gases, and bio-oils (Demirbas 2007).

Bio-oils have been used sparingly due to their incompatibility with standard fossil fuels; however, catalytic upgrading should enhance their acceptance. For lignin valorisation, gasification is an important method. It emits hydrogen (H2), carbon monoxide (CO), and a trace amount of carbon dioxide (CO_2). The most technologically advanced region is the use of syngas. Syngas can be used in a variety of applications. Processes for producing DME (methanol/dimethyl ether) and Fischer-Tropsch for producing green diesel, for example, are well-established. Syngas may also be used for cooking, heating, and power generation. Hydrolysis, polymerisation, hydrogenation, and methanation are all steps in the complete gasification of lignin (Figueiredo et al. 2018).

19.7.3 Oxidised Products

The oxidation of lignin produces aldehydes, vanillin, vanillic acid, dimethyl sulphoxide (DMSO), aromatic acids, aliphatic acids, and cyclohexane. Organometal catalysed oxidation, biomimetic oxidation, enzyme-based oxidation, and organic solvent nanofiltration are some of the methods for oxidising lignin that have been adopted (Lange et al. 2013; Werhan et al. 2012). It is susceptible to oxidation due to the presence of a phenolic group. Oxidants such as nitrobenzene, metallic oxides, and hydrogen peroxide are widely used to maintain the aromatic character. Vanillin, an aldehyde, is the most important ingredient of natural vanilla extract. It is used as a flavouring agent in fruits, drinks, and pharmaceuticals.

Aromatic acids, aliphatic acids, and cyclohexanol are some of the other lignin oxidised compounds.

19.7.4 Polyhydroxyalkanoates

PHA is a group of biopolyesters that are synthesised as an energy reserve within cells and can be generated by a variety of microbes when nutrients are depleted. PHA has received a lot of attention as a biodegradable plastic in the last three decades, not just because of its good material consistency, but also because it can be produced from sustainable carbon sources, including its low biomass constituents (Madkour et al. 2013). PHA can be produced by a variety of bacteria from lignin-based aromatics (Linger et al. 2014; Si et al. 2018; Kumar et al. 2017; Tomizawa et al. 2014; Zhang et al. 2018; Wang et al. 2018). In addition to aromatic monomers, certain genuine lignin streams may be used for PHA biosynthesis. By combining PHA production with chemical catalysis, the lignin valorisation path was lengthened, allowing for a wider range of produced PHA. First, associated microorganisms converted lignin stream or pretreated lignocellulosic liquor to PHA.

19.7.5 Lipid

Lipids are attractive feedstocks for the manufacture of biofuels. In general, oleaginous species can store lipids at a rate of more than 20% of their dry cell weight (DCW). Certain oleaginous microbes have recently been discovered to be able to synthesise lipid from lignin-based aromatics. Various lignin-based aromatics can be metabolised by *Rhodococcus* bacteria. In addition to lignin residuals, aqueous wastes containing lignin-based aromatics can be used for lipid processing with bacteria.

A large amount of waste effluent could be produced during the pretreatment of lignocellulose, which requires different fractions depending on the pretreatment process. Lipid-producing microorganisms can use alkali pretreatment waste effluent containing lignin, aromatics, oligosaccharides, and carbohydrates for the production of a wide array of lipid compounds (He et al. 2017).

19.7.6 Aromatics

Natural aromatics are gaining popularity as a result of their numerous applications. Lignin is an outstanding feedstock for natural aromatics because of its unusual aromatic structure and repeatable properties.

Only three aromatics have been obtained from lignin using biological methods: vanillin, p-hydroxybenzoic acid, and pyrogallol. The primary organoleptic ingredient in vanilla pods is vanillin, which is commonly used in cooking, cosmetics, pharmaceuticals, and other industries.

The demand for natural products has spurred extensive research into biological processes that use fungi, bacteria, and plant cells to produce vanillin from glucose, phenolic stilbenes, isoeugenol, eugenol, or ferulic acid.

19.8 Conclusion

Lignin is one of the world's most plentiful renewable resources, but it's often ignored when it comes to valuing lignocellulosic biomass. Lignin-based materials and chemicals have received a lot of press as a potential biofuel and bio-based product feedstock. While substantial progress has been made, lignin's high recalcitrance and heterogeneity have created several major problems that must be addressed in order to increase lignin processing. Despite the myriad issues with lignin valorisation, a number of solutions have emerged that could result in high-yielding value-added goods. A microbial cell factory inspired by nature's complex collection of lignin-degrading microbes is at the core of these innovative procedures. Lignin conversion through biological platform employs the catalytic ability of microorganisms to decompose complex lignin molecules and generate value-added products via biosynthesis. Ligninolytic microorganisms provide a wide range of enzymes for dissolving lignin polymer linkages, as well as peripheral pathways for converting heterogeneous lignin derivatives to core intermediates and target items. During lignin depolymerisation, lignin oxidation and recondensation of damaged fragments occur concurrently; preventing degraded lignin from repolymerisation is crucial for maximising monophenolic compound yield. For the manufacture of value-added chemicals, biological lignin degradation is preferable to thermochemical processes since it is more environmentally safe. It is anticipated that genetically modified microorganisms will be a more viable approach to produce high-yield commodity chemicals than other methods, which either have high maintenance costs or are not environmentally friendly.

Acknowledgements The authors are thankful to UGC SAP project no. F.3-14/2016/DRS-I (SAP-II) and Chhattisgarh Council of Science and Technology (CCOST), Raipur, India (sanction order no: 2142/MRP/2015).

Declaration All the authors declare that they have no competing interests.

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Bioremediation Technology: A Cumulative Study on Microbial Bioremediation of Heavy Metals, Aromatic Hydrocarbons, Acrylamide, and Polyacrylamide

20

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Abstract

Hydrocarbons, heavy metals, and xenobiotics represent the biggest cluster of environmental pollutants worldwide. Upon release into the environment, they undergo a complex physicochemical transformation into more recalcitrant and even more toxic products. Majority of the reports showcase their toxicity and implications in marine, air, and soil environments. Due to their poisonous, carcinogenic, and mutagenic properties, and their pervasive production, recalcitrance, as well as environmental persistence, PAHs, heavy metals, and PAM have tremendous public interest and crucial environmental problems across the globe. Awareness of the detrimental consequences, along with human well-being, has contributed to researchers' interest in the degradation of these compounds using bioremediation technology, which appears to be the most acceptable and economically viable approach. Remedial strategies need reliable methods to identify and monitor contaminations, as well as effective procedures to attenuate or eliminate from the environment for the sustained ecosystem. A comprehensive compilation of eco-friendly biological treatment methods for the remediation of hydrocarbons and heavy metals, such as microbial remediation approaches using microbes, is given in this study. A summary of innovative technologies is presented, and new ideas on value-added by-product generation during such remediation process are illustrated in this study.

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_20

Keywords

Bioremediation · Ex situ · Bioavailability · Toxicities

Abbreviations

AA	Acrylamide
FTIR	Fourier transform infrared spectroscopy
GAMA	*N*-acetyl-S-(2-carbamoyl-2-hydroxy-ethyl)-L-cysteine
GC-MS	Gas chromatography-mass spectroscopy
GSH	Glutathione
GST	Glutathione S-transferase
HbAA	*N*-(2-carbamoylethyl)valine
HbGA	*N*-(2-carbamoyl-2-hydroxyethyl)valine
HPLC	High-performance liquid chromatography
IARC	International Agency of Research on Cancer
Iso-GAMA	*N*-acetyl-S-(1-carbamoyl-2-hydroxy-ethyl)-L-cysteine
LC-MS	Liquid chromatography-mass spectroscopy
PAH	Polyaromatic hydrocarbons
PAM	Polyacrylamide
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Nrf-2	Nuclear factor erythroid 2
TNF	Tumor necrosis factor
OD	Optical density
SPE	Solid-phase extraction

20.1 Introduction

Contamination of the environment has rapidly increased due to urbanization and industrialization. Industries play important role in the world economy and society; however, they have made a lot of negative impacts on the biosphere (Manisalidis et al. 2020). Environmental pollution has emerged as a global problem that is common to both developed and developing countries with severe long-term consequences. The decline in environmental quality as an outcome of pollution is corroborated by the loss of biological diversity from an excessive amount of harmful chemicals released in the environment resulting in growing risks of environmental accidents and threats to biota (Hunter 2007). Air, water, and soil pollution is a current as well as a future problem worldwide. Though apparently looking economically significant, these compounds have become a consequential problem for the planet.

Microbial bioremediation is defined as a process to degrade, break down, or transform hazardous contaminants into less toxic or nontoxic forms by either naturally occurring or genetically engineered microbes or microbial consortia, thereby eliminating contaminants from the environment (Agrawal and Verma 2020a; Agrawal et al. 2020a). These contaminants are used as an energy source by microorganisms in their metabolic processes (Azubuike et al. 2016). Bioremediation has gained momentum as a tool for environmental cleaning in the last two decades. Various bioremediation mechanisms have become the epicenter of degradation approaches such as use of a variety of microorganisms to clean contaminated environments taking into account the unique mechanism each organism possesses for decontaminating the environment based on their physiological systems and metabolic capabilities (Ojuederie and Babalola 2017; Agrawal et al. 2020b; Kumar et al. 2019). The bioremedial approaches are generally classified into two types: in situ and ex situ. Ex situ bioremediation is the process in which the contaminated soil is excavated to be moved to another site for the intended treatment, thereby increasing the cost of the process. On the other hand, in situ bioremediation is the sustainable process whereby contaminants are degraded biologically under natural conditions to simpler transformed products leading this to be a low cost, low maintenance, and environment-friendly approach for the cleanup of contaminated soils. In situ bioremediation refers to the process of decontamination of an area where the contaminants are treated "at location" without physically displacing the contaminated material (Megharaj and Naidu 2017). It is preferred as it offers a balance between cost-effectiveness and site buildability. This technology depends on various parameters such as the interaction between site soils, microbiology, water chemistry, contaminant contamination, and physiological conditions such as site pH and temperature (Ayangbenro and Babalola 2017). The main concern in the type of bioremediation to be implemented is the process of determining whether the site environment is appropriate for in situ bioremediation. In situ bioremediation can be achieved via the following of three approaches: (1) bioaugmentation, where microbes with known degrading capabilities are added to the contaminated site; (2) bioattenuation, which is the process dependent on the natural process of degradation; and (3) biostimulation, which is defined as the stimulation of bioremediation achieved by the addition of nutrients and electron donors or acceptors. Bioaugmentation can be accomplished via two strategies: (1) isolation of indigenous microbes from the contaminated soils, enriching them under laboratory conditions and returning them to the contaminated site, or (2) inoculation of nonindigenous microorganisms (from different contaminated sites) with reported abilities to degrade the contaminants of concern. Bioattenuation relies on natural processes for the biological transformation of contaminants, during which the indigenous microbial populations based on their metabolic processes degrade recalcitrant or xenobiotic compounds (Bisht et al. 2015). The process includes a combination of chemical, physical, and biological processes aimed at reducing the toxicity or concentration of contaminants. Biostimulation is the process of adding nutrients to a contaminated site in order to induce the growth of indigenous microorganisms along with macro- and micronutrients capable of degradation of the target molecule

(Sarkar et al. 2016). Certain stable organic supplements such as compost, sewage sludge, manure, etc., are also added as biostimulants for the activation of innate catabolic pathways of microorganisms (Yakhin et al. 2017). Bioaugmentation is the technique of introduction of specific strains or a consortium of microorganisms to the contaminated site aimed at improvement in the removal capacity of contaminated areas, thus favoring the biodegradation process.

However, for the restoration of large-scale contaminated land, in situ approach is preferred to ex situ remediation. The use of bioremediation technology has been known to be dependent on several factors such as contaminated site conditions, use of microbial strains and their respective activities, quantity, and toxicity of the contaminant species present (Ayangbenro and Babalola 2017). Microbial bioremediation is the natural way to mitigate environmental pollution including the activities of aerobic and anaerobic microorganisms. The common strategy that is used for applying microbes in such a scenario is their difference in nutritional requirements.

This chapter confabulates on the toxicity caused by contamination of heavy metals polyhydroxy aromatic hydrocarbons (PAHs), acrylamide and polyacrylamide, and functioning of biological remediation means to emancipate their toxic effects.

20.2 Heavy Metal as a Source of Environmental Pollution

The rapid urbanization in the recent past has led to a substantial surge in the pollution level of toxic wastes of heavy metals such as copper, iron, manganese, and zinc which are released through tannery industries wastewater and chromium and nickel in untreated effluents by electroplating plants (Kapahi and Sachdeva 2019; Pratush et al. 2018). The sorority between the use of heavy metals on a daily basis and the development of heavy metal resistance in the environment has been persisting for decades (Sütterlin et al. 2014). The upstanding concentrations of heavy metals enter the environment through manual processes of metalliferous mining, smelting, and disposal of metallic wastes (Das and Tiwary 2013; Kang et al. 2016; Prithviraj et al. 2014). They have been used unconditionally and uncontrollably in the healthcare sector as well as antiseptics and disinfectants (Hobman and Crossman 2015), apart from being the major constituent of industrial wastes, like solvents, chemicals, weeds, etc. (Mukhopadhyay et al. 2002; Flanagan et al. 2012), contributing to environmental pollution, with amplitude of ill effects to flora and fauna. Heavy metals such as zinc and copper which are used for growth and therapeutic purposes in pig and poultry farms get accumulated in the soil and water environment. The human microbiome, especially gut microbiota, is divulged to these ill effects through consumption of groundwater and other various sources (Silver 2003; Hojsak et al. 2015). Heavy metals such as arsenic (As), lead (Pb), mercury (Hg), chromium (Cr), zinc (Zn), cadmium (Cd), copper (Cu), and nickel (Ni) are recognized pollutants. The limited concentration of heavy metals is requisite for appropriate metabolism in microorganisms, but when the quintessential concentration gets exceeded, it results in the drastic botheration to the whole environment.

Cd is known to cause many ill effects on the respiratory tract apart from cardiovascular and renal; Cr is reported as a major factor for causing a mental disturbance, cancer, ulcer, and hypoketotosis. Cu is reported to provoke anemia; Pb is notable in inducing neurotoxic effects on humans; Ni incites skin allergies and lung fibrosis, and Zn elicits abdominal pain, nausea, vomiting, diarrhea, irritability, lethargy, and anemia (Pratush et al. 2018; Kumar et al. 2021).

Scientists have reported a multitude of methods for the removal of heavy metals from contaminated sites, which may include ion exchange, dialysis, reverse osmosis, solvent extraction, and chemical precipitation. However, these techniques are the least cost-effective and are not competent enough, apart from downgrading the quality of soil (Xu et al. 2017). Strategies of bioremediation have proved to be very eco-friendly, as they are executed using microbes, plants, and fungi. The mode of their mediation mostly relies on the sorption of heavy metals and transforming them by changing their valance state and hence reducing the toxicity (Ayangbenro and Babalola 2017). Bacteria that are reported for heavy metal transformation include species from the genera *Acinetobacter*, *Aeromonas*, *Brevundimonas*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Geobacter*, *Streptomyces*, *Arthrobacter*, and *Cyanobacteria*. Apart from bacteria, fungi such as *Aspergillus*, *Penicillium*, *Rhizopus*, and *Gloeophyllum* and yeast such as *Rhodotorula*, *Saccharomyces*, and *Candida* are also divulged (Pratush et al. 2018).

20.2.1 Mechanism of Bioremediation of Heavy Metals

The accretion of heavy metals is catastrophic to the environment as metals have the tendency to get accumulated in the environment as their most stable oxidation states, i.e., As^{+3}, Pb^{+2}, Hg^{+2}, and Cd^{+2}, which in turn generate acutely resistant biotoxic compounds, when reacted with biomolecules of environment and become very difficult to dissociate (Kulshreshtha et al. 2014). The different strategies involved include changing the ionic states of heavy metals which enhances their bioavailability, thus augmenting processes that improve bioremediation (Ayangbenro and Babalola 2017). Mobilization and immobilization constitute the fundamental process of heavy metal treatment by microorganisms.

Mobilization includes redox reactions which involve tasks such as dissolving the heavy metals and lowering the pH. Mobilization is further classified into subprocesses such as enzymatic oxidation, enzymatic reduction, complexation, and siderophores. Enzymes released from microbes which catalyze the enzymatic oxidation of heavy metals augment its solubility by oxidizing higher state to lower state. This method removes inorganic heavy metals from the sites of contamination. This method ensures the transformation of heavy metals to less toxic form by loss of electron or oxidation of higher state to lower state, as reported for *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* for biotransformation of uranium (Cumberland et al. 2016). The other method of mobilization, termed enzymatic reduction, is a complete reverse of enzymatic oxidation. This method pulls out such compounds which have multiple oxidation states and remains insoluble in

their reduced state and completely remediate them. Microbial reduction for decontamination is reported for remediation of heavy metals such as mercury and chromium (Jouety et al. 2015; Sinha et al. 2009) This action is performed mostly by facultative and obligate anaerobes (Rabus et al. 2016). Some decontamination processes involve both enzymatic oxidations as well as reduction. For example, arsenic is both oxidized and reduced to As^{5+} from As^{3+}. Oxidation is carried out by enzyme arsenic oxidase, whereas during reduction, either cytoplasmic or periplasmic way is applied. The reduced As^{3+} gets accumulated in the intracellular compartments as free arsenite or may conjugate with glutathione or other thiols (Satyapal et al. 2016). Similarly, additionally, the process of complexation, which works on the same mechanism of mobilization, adds ligands to the inorganic metals, thus creating a complex. This metal-ligand complex establishes the removal of heavy metals from solid wastes. Two types of complexing channels are reported, viz., high molecular weight ligands and low molecular weight ligands, which include chelators such as citric acid, tricarboxylic acid, and alcohols. High molecular weight ligands include siderophores also known as specific iron chelators in iron-deficient medium. Siderophores are noticed to form complexes with heavy metals and boost their solubility (Khan et al. 2017).

Immobilization techniques are used in the case of in situ bioremediation, wherein the treatment for remediation is at the contaminated site itself. The process is structured by factors such as soil bacteria, soil moisture, and temperature. Immobilization methodology is prorated into active and passive methods. Passive immobilization is an energy-independent method and isn't specific on the metal, whereas active immobilization which is energy dependent relies upon microbial metabolism, thus making the process curbed. The immobilization method is executed using methods such as precipitation, biosorption, and bioaccumulation (Pratush et al. 2018), for example, lead and mercury remediation. In precipitation, metal ions are precipitated by chemical reduction and removed from the contaminated sites. After precipitation, metal's toxicity is almost nullified (Martinez et al. 2014). Biosorption is another mode of bioremediation in which metal ions are absorbed by bacteria or other microbes, thereby reducing their concentration in contaminated sites. It is a cost-effective solution, as cellulose which is commonly used as sorbents is easily available (Malik et al. 2017). Researchers have reported the use of extracellular polymeric substances (EPS), a mixture of polysaccharides, mucopolysaccharides, and proteins, as sorbents for toxic metal ions. Peptidoglycans and phosphate groups constitute the main cationic and anionic sites for biosorption for bacteria. However, for fungi, chitin, phenolic polymers, and melanin establish the biosorption sites. It is a cost-effective, no drawback, no sludge forming, reproducible method. It can be performed two ways: single or double step. In the single-step biosorption process, the biosorbent microbial species are incubated with a medium contaminated with heavy metal in a mixing tank, and after the full growth of microbes, the solution is filtered out. However, in double-step biosorption, the filtrate obtained of single-step again undergoes incubation with the biosorbent species, making it much more competent (Pratush et al. 2018). Bioaccumulation is the accumulation of heavy metals by microorganisms. The accumulation is triggered by activities of ion

pumps, ion channels, endocytosis, and lipid permeation (Satyapal et al. 2016). It is considered as one of the most fortuitous immobilization techniques in the recent past, with successful bioaccumulation of metals such as mercury, lead, silver, cadmium, nickel, cesium, cobalt, chromium, and uranium (Olaniran et al. 2013). Table 20.1 summarizes the occurrence, toxicity, mode, and microorganisms involved in the bioremediation of heavy metals.

20.3 Acrylamide and Polyacrylamide as Source of Environmental Pollutant

Acrylamide (AA) (C_3H_5NO) is an organic white crystalline solid with a melting point of 125 °C. Polyacrylamide (PAM) is a water-soluble polymer derived from the monomer acrylamide. Most of the AA produced is used as a chemical intermediate or as a monomer in the production of PAM. AA and PAM are used in diverse industrial processes, mainly the production of plastics, dyes, and paper and in the treatment of drinking water, wastewater, and sewage (Okaiyeto et al. 2016). AA polymers have also found their application as an additive to enhance oil recovery from underground, increase dry strength of paper products, fog dissipation, and soil stabilizer. Due to its highly reactive and water-soluble nature, AA has diverse chemical and environmental applications such as in the production of paper, dyes, plastics, etc. (Benkhaya et al. 2020). PAM can be manufactured as a cationic, anionic, and nonionic polymer through a free-radical mechanism in an aqueous solution. AA and its derivatives are used as flocculants in chemical and food processing industries where it is used to flocculate the suspended matter (contaminants) present in water used for irrigation and drinking (Maćczak et al. 2020). AA has been found as a component of the effluents from the dyes, organic chemicals, pesticides, and plastics industries (Thompson and Darwish 2019). This contaminant rapidly moves down to groundwater via seepage eventually increasing exposure risk on public health. Over the past two decades, AA has emerged as a molecule of concern in food and environmental science owing to its carcinogenic effect on human health. In the year 2002, a group of Swedish scientists found the formation of AA when cooking carbohydrate-rich foods at temperatures beyond 120 °C (Paul and Tiwari 2020; Virk-baker et al. 2014). This discovery of AA formation via Mailard reaction was found to be dependent on factors such as time, carbohydrate to nitrogen concentration, and temperature (Miśkiewicz et al. 2020). Since then, the world has focused on scientific studies focused on its toxicology and control. The International Agency of Research on Cancer (IARC) in 1994 classified AA as a "2A Group" carcinogen, exposure to which can cause neurological anomalies (Fernandes et al. 2019). PAM has been found to be nontoxic to the biota; however, its monomeric form, i.e., acrylamide, has been linked with neurotoxicity although it is biodegradable and does not accumulate in the soil. The major source of AA that is released into the environment is from food, beverage, paper, oil, pharmaceutical industries.

Table 20.1 The occurrence, toxicity, mode, and microorganisms involved in the bioremediation of heavy metals

Heavy Metals	Source of contamination	Effects	Mode of remediation	Microorganisms reported for Bioremediation	References
As	Smelting, metal refining, coal combustion, soil erosions, and volcanic eruptions	As poisoning, diabetes, carcinogenic, neurobehavioral disorders, leukonychia, stroke, chronic lower respiratory disease, night blindness	Oxidation, coagulation, precipitation, filtration, adsorption, ion exchange, membrane technologies	*Bacillus aryabhattai*, *Brevibacillus brevis*, *Scopulariopsis brevicaulis*, *Geospirillum arsenophilus*, *Geospirillum barnesi*, *Desulfutomaculum auripigmentum*, *Bacillus arsenicoselenatis*, *and Crysiogenes arsenatis*, *Gallionella ferruginea*, and *Leptothrix ochracea*	Flanagan et al. (2012); Hojsak et al. (2015); Mukhopadhyay et al. (2002); Satyapal et al. (2016)
Pb	Mining, smelting, glass manufacturing, lead batteries, coal combustion, paints, soldered metal products	Pb poisoning, brain damage, retardation, infertility, anemia, seizures, coma	Biosorption, complexation, precipitation, bioaccumulation, rhizofiltration	*Pseudomonas marginalis*, *Klebsiella aerogenes*, *Cupriavidus metallidurans*, *Bacillus megaterium*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Alcaligenes eutrophus*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Citrobacter freundii*	Pratush et al. (2018); Rabus et al. (2016)
Hg	Mining, painting, electrical batteries, switches, petrochemicals, fertilizers and fungicidal sprays, dental amalgam	Hg poisoning, Minamata disease, acrodynia, kidney problem, decreased intelligence	Autotrophic leaching, heterotrophic leaching, methylation, redox transformation	*Pseudomonas putida*, *Cupriavidus metallidurans*, *Lecythophora*, *Triticum aestivum*, *Glutamicibacter*	Prithviraj et al. (2014); Olaniran et al. (2013)

				sp., *Bacillus* sp., *Planomicrobium* sp., *Bergeyella* sp., *Brevundimonas* sp., *Ochrobactrum* sp., *Glutamicibacter* sp., *Brevundimonas* sp., *Ochrobactrum*	
Cd	Welding, mining, coating, plating, plastics, combustion of fossil fuels and municipal wastes, production of alloys and batteries	Cd poisoning, cancer, respiratory damage, tracheobronchitis, pneumonitis, pulmonary edema, anemia, kidney failure	Bioaccumulation, biosorption, ion exchange, filtration	*Bacillus* sp., *Pseudomonas aeruginosa*, *Citrobacter* sp., *Phormidium valderium*, *Chlorella vulgaris*, *Aspergillus niger*, *Pleurotus ostreatus*, *Rhizopus arrhizus*, *Escherichia coli*, *Pseudomonas* sp., *Comamonas testosteroni*, *Klebsiella planticola*, *Staphylococcus aureus*, *Bacillus subtilis*, *Lactobacillus plantarum*, *Staphylococcus lugdunensis*, *Ralstonia metallidurans*, *A. eutrophus*, *Serratia liquefaciens*, and *Bacillus thuringensis*	Martinez et al. (2014); Xu et al. (2017)

(continued)

Table 20.1 (continued)

Heavy Metals	Source of contamination	Effects	Mode of remediation	Microorganisms reported for Bioremediation	References
Cr	Welding, metallurgy, wood preservation, metal processing, dyes, paints, leather tanning	Hemotoxic, genotoxic, carcinogenic, hemolysis, kidney and liver failure	Direct and indirect bioreduction, anaerobic bioreactor utilizing marine-derived sulphate-reducing bacteria, immobilized as a biofilm on a gravel, biosorption	*Comamonas testosterone*, *Klebsiella planticola*, *Staphylococcus aureus*, *B. subtilis*, *Lactobacillus plantarum*, *Staphylococcus lugdunensis*, *Ralstonia metallidurans*, *A. eutrophus*, *Serratia liquefaciens*, *Bacillus thuringensis*, *Enterobacter cloacae*	Jouety et al. (2015); Malik et al. (2017)
U	Mostly geogenic, mining and milling, combustion, corrosion, nuclear waste	Cancer, leukemogenic, genetic, reproductive, and neurological effects, birth defects	Biosorption, bioaccumulation, bioprecipitation	*Synechococcus elongatus*, *Anabaena torulosa*, *Serratia marcescens*, *Acidithiobacillus ferrooxidans*, *Deinococcus radiodurans*, *Desulfovibrio desulfuricans*, *Shewanella putrefaciens*	Cumberland et al. (2016); Kapahi and Sachdeva (2019)

AA is a highly reactive agent that can react with hydroxyl-, amino-, and sulfhydryl-containing compounds spontaneously (Stevens and Maier 2008). Humans can be exposed to AA via three routes: oral, dermal, and inhalation; thus, the study of "human acrylamide exposure" comprises of a combination of diet, second-hand smoke exposure, drinking water, occupational sources, and personal care items (Virk-baker et al. 2014). Potable water sources have been known to contain biological contaminants such as bacteria, fungi, algae, and organic and inorganic materials which are known to possess their native charges (Cabral 2010; Prest et al. 2016; Novak Babič et al. 2017). For the remediation and purification of the water, these charged particles are aggregated using metal salts as coagulants which neutralize the surface charges of the particles. Also, PAM is known to be widely utilized in the remediation of municipal sewage effluents for separating, settling, thickening, and dewatering solids wastes. The use of anionic, cationic, as well as nonionic PAM in the purification of industrial wastewaters with suspended fine solids has also been reported (Maćczak et al. 2020).

The biodegradation and environmental fate of acrylamide in water, soil, and food products have been examined. Early researchers such as Cherry et al. (1956) and Croll et al. (1974) found that AA degraded in filtered river water in 10–12 days whereas in river water ~4 days. Lande et al. (1979) found that complete degradation of acrylamide in the soil to carbon dioxide occurred in ~6 days.

20.3.1 Acrylamide Metabolism in Living Beings

A substance is said to be toxic for a biological system when it has the potential to cause an imbalance in the biological oxidant to antioxidant ratio causing oxidative stress ultimately leading to many diseases. Due to this oxidative stress, the body has a mechanism of continuously counteracting the adversities. In recent years, studies demonstrated the oxidative stress induced by acrylamide affecting the cellular redox chain with a potential to generate reactive oxygen species (ROS) (Nowak et al. 2020). As the free radicals are constantly produced in our bodies which are also known to have adverse effects, the body produces antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione *S*-transferase (GST), glutathione peroxidase, and proteins like glutathione (GSH) to counteract the adversities (Lobo et al. 2010). AA is initially oxidized to glycidamide, both of which interact with nucleophiles groups such as –SH, –NH2, or –OH present inside the cells. AA reacts with GSH by helping of glutathione S-transferase. In response to the oxidative stress from the increase in AA concentration within the body, GST, SOD activity, and apoptosis are increased with a decrease in GSH count (LoPachin and Gavin 2016). Once consumed, due to its low molecular weight and water-soluble nature, acrylamide is absorbed by the gastrointestinal tract via circulation and passive diffusion and is distributed to peripheral tissues such as the heart, thyroid, stomach, kidney, liver, and testis (Kumar et al. 2018). Researchers also found the presence of AA in breast milk along with the possibility that acrylamide could pass through the placenta and reach the developing fetus (Duarte-Salles et al. 2013). AA is metabolized in the

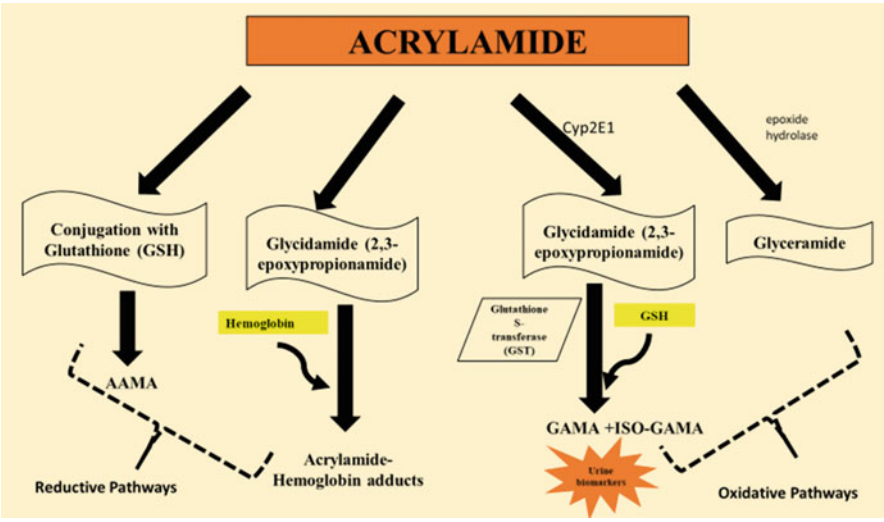

Fig. 20.1 Summary of acrylamide metabolism in humans

human body via four major pathways: (1) conjugation with reduced glutathione (GSH) to form GSH adducts, (2) conjugation with hemoglobin and DNA molecules in the bloodstream, (3) conversion to a chemically reactive epoxide, glycidamide, in a reaction catalyzed by the cytochrome P450 enzyme complex Cyp2e1 which further reacts with GSH to form mercapturic acid metabolites excreted in urine, and (4) formation of glyceramide from glycidamide (Duda-Chodak et al. 2016) (Fig. 20.1). One of the oxidative metabolisms starts with the conversion of AA to glycidamide (2,3-epoxypropionamide) mediated by the enzyme Cyp2e1.

Glycidamide has been shown to bind with hemoglobin and DNA to form hemoglobin-glycidamide and DNA-glycidamide adducts, respectively. Glycidamide can also combine with GSH in the presence of glutathione S-transferase (GST) leading to the formation of two mercapturic acid metabolites, viz., *N*-acetyl-S-(2-carbamoyl-2-hydroxy-ethyl)-L-cysteine (GAMA) and *N*-acetyl-*S*-(1-carbamoyl-2-hydroxy-ethyl)-L-cysteine (iso-GAMA) (Obón-Santacana et al. 2016). These two metabolites are excreted through urine and are considered to be biomarkers. The other oxidative metabolic pathway takes up acrylamide as the substrate and converts it to glyceramide in the presence of epoxide hydrolase enzyme (Ghanayem et al. 2005). On the other hand, one reductive metabolic pathway entails the direct conversion of AA combined with GSH to form adducts like *N*-acetyl-*S*-(1-carbamoyl-2-hydroxy-ethyl)-L-cysteine (AAMA). Another reductive pathway occurs in the bloodstream where acrylamide directly combines with hemoglobin to form Hb-acrylamide adducts such as *N*-(2-carbamoylethyl)valine (HbAA) and *N*-(2-carbamoyl-2-hydroxyethyl)valine (HbGA), which also serve as internal biomarkers (Fennell et al. 2004).

20.3.2 Acrylamide-Mediated Toxicity

Although most human exposures to AA result from dermal absorption or ingestion of dust, one report documented exposure and toxicity resulting from drinking water contaminated with AA. In 1975, it was reported that five members of a Japanese family who have ingested well water contaminated with acrylamide reported the AA concentration of approximately 400 mg/L (Igisu et al. 1975). The implications of this toxicity were found to be varying among the children and the adults; the adults who exclusively consumed AA-contaminated water displayed abnormalities such as slurred speech, unsteady gait, memory loss, irrational behavior, visual, tactile, and auditory hallucinations as compared to the children who partly consumed acrylamide-free water while at school and developed mild gait disorders and sleep disorders. Reports have also suggested that the neuropathy induced by AA toxicity has symptoms such as slowly progressing symmetric distal sensory abnormalities and motor weakness (Pennisi et al. 2013). However, clinical reports also suggest that patients with severe neuropathy may never completely recover but may experience residual ataxia, distal weakness, and sensory loss (Kim et al. 2017). AA has been known to be rapidly distributed to all tissues where both AA and glycidamide bind to sulfhydryl groups and extracellular amino acids and/or proteins which enables AA to have various implications on the body which can either be carcinogenic or noncarcinogenic in nature (Rice 2005). The apoptotic effect of AA has been well documented with several possible molecular mechanisms. As explained earlier, oxidative stress results in the destruction of cellular macromolecules and ultimately apoptosis which is the process of regulated cell death and occurring through various anomalies such as apparent cell shrinkage and fragmentation, genomic DNA cleavage, and cellular condensation (Miller and Zachary 2017). AA acts as an electrophile and interacts with the nucleophilic residues of DNA and several other cellular proteins causing different physiological alterations within the cell (LoPachin and Gavin 2016). It has been known to inhibit cell proliferation as it interferes with the functioning of kinesin proteins, responsible for the spindle fibers formation during cell division. The oxidative stress induced by acrylamide in the cell causes the activation and transfer of nuclear factor erythroid 2-related factor 2 (Nrf2) into the nucleus, further activating antioxidant protective genes (including heme oxygenase-1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1), GST, TNF-a, colony-stimulating factor, and interleukins such as IL-1b and IL-6) as counteraction (Zhang et al. 2013). The activation of nuclear factor jB (NF-jB) in response to ROS triggers the secretion of pro-inflammatory cytokines such as IL-1b, IL-6, and tumour necrosis factor-a which are secreted, leading to an inflammatory response against AA (Eda et al. 2011). The secretion of such proteins activates apoptosis-related proteins such as p21, p27, and p53 which are known to stop G_0/G_1 phase in cell division.

20.3.3 Approaches for PAM/Acrylamide Bioremediation

At the advent of the twenty-first century and the Industrial Revolution, the occurrence of environmental pollution caused by various contaminants leads to seeking a sustainable and eco-friendly process that does not add to the environmental adversities significantly. Biodegradation of a contaminant is said to be dependent on three factors: (1) the ability of microorganisms to degrade a specific chemical structure; (2) external factors such as pH, temperature, humidity, aeration, nutrient content, redox potential, etc., which contribute to the microorganisms' ability to express degradation enzymes; and (3) homeostasis between the organism and the organic substrate (Mohanan et al. 2020). Due to its reactive nature, acrylamide may not be completely degraded in industrial effluents and water treatment facilities; hence biodegradation serves as one of the methods for removal of contaminants to an acceptable concentration that is below the limits established by the regulatory agencies. Based on laws of thermodynamics, a compound of organic origin can serve as an energy source for aerobic microorganisms. Exploiting this approach, many researchers have utilized microorganisms for the bioremediation of polyacrylamide. Bioremediation also turns out to be a less invasive approach to the environment as it prevents secondary pollution and keeps the environment intact. Researchers have devised effective bioremediation approaches especially using microbes capable of growth by degrading the targeted toxic contaminants. Microbes isolated from various sources have been extensively used to degrade or transform acrylamide under certain environmental factors like high temperatures or UV light. Literature suggests that utilizing AA/PAM as the sole carbon and/or nitrogen source for growth and metabolism is harder due to the availability of appropriate enzymes (Haveroen et al. 2005). Well-known carbon sources like glucose and fructose have been reported to be used as support elements or additional carbon sources in a growth medium containing AA/PAM for the growth and degradation of AA-degrading bacteria (Shukor et al. 2009). Researchers have also found that PAM could serve as the sole source of nitrogen, due to the presence of extracellular enzymes capable of hydrolysis of amide group to ammonia, such as amidases (Yu et al. 2015). PAM bioremediation commences with its deamination by an amidase enzyme to polyacrylate and ammonia which is then utilized as a nitrogen source for growth by the microbes (Duda-Chodak et al. 2016). Subsequently, the main carbon backbone of PAM is cleaved in the presence of monooxygenases and transformed into smaller compounds, such as acrylamide and acrylic acid, which could be further degraded or utilized in various catabolic pathways (Joshi and Abed 2017).

Since the early 1980s, AA degradation via microbes has been explored extensively, mainly with *Bacillus*, *Pseudomonas*, *Enterobacteriaceae*, and *Rhodococcus* isolates. (Table 20.2). In the past decades, researchers have explored the concept of immobilized cells for acrylamide removal because of the presence of a coating substance that proves to be a shield against the adversities such as predators, toxins, or parasites, hence saving resources and time. However, the efficiency of the immobilized cells may be dependent on pH, temperature, and initial AA

Table 20.2 Microbes reported for degradation of acrylamide and polyacrylamide

Microbial strains	Source	Substrate	Conditions	End product	Technique used	Biodegradation %	Reference
Acinetobacter sp. *no. 11*	Curing pot for HPAM distribution center	Hydrolyzed polyacrylamide	Anaerobic	Acrylamide oligomer derivatives	GC-MS	30.8	Ma et al. (2008)
Azomonas macrocytogenes Enterobacter agglomerans	Activated sludge/soil	PAM	Aerobic	NA	Column chromatography	20	Nakamiya and Kinoshita (1995)
Pseudomonas sp. *strain DRYJ7*	Antarctica soil	Acrylamide	Aerobic/25 °C	Acrylic acid and ammonia	HPLC	90	Shukor et al. (2009)
Bacillus cereus HWBI Bacillus flexus HWBII	Activated sludge/soil	PAM	Aerobic/30 °C; PAM and glucose as carbon source	Acrylamide, other metabolites	FTIR	70	Wen et al. (2010)
Saccharomyces cerevisiae	Supermarket	Acrylamide hydroxymethyl furfural	Aerobic/30 °C; HMF, acrylamide, sucrose as carbon source	–	UFLC-MS	99.2	Akillioglu and Gökmen (2014)
Enterobacter aerogenes	Domestic wastewater	Acrylamide	Aerobic	Acrylic acid, ammonium	HPLC	94	Buranasilp and Charoenpanich (2011)
Free and immobilized P. aeruginosa	Soil	Acrylamide	Aerobic/20 °C	Ammonia/ CO_2	HPLC	NA	Prabu and Thatheyus (2007)

(continued)

Table 20.2 (continued)

Microbial strains	Source	Substrate	Conditions	End product	Technique used	Biodegradation %	Reference
Ralstonia eutropha AUM-01	Soil	Acrylamide	Aerobic	Acrylic acid	Optical density	NA	Cha and Chambliss (2013)
Kluyvera georgiana	Domestic wastewater	Acrylamide	Aerobic/37 °C	Acrylic acid	HPLC	92.3	Thanyacharoen et al. (2012)
B. cereus	Oil field	Partially hydrolyzed polyacrylamide	Aerobic/37 °C	Smaller molecules	FTIR, HPLC	100	Bao et al. (2010)
Moraxella osloensis MSU11	Paper mill effluent	Acrylamide	Aerobic/30 °C	Acrylic acid	Optical density	NA	Jebasingh et al. (2013)
Pseudomonas aeruginosa DS-4	Diesel contaminated soil	Acrylamide	Aerobic	–	Optical density	–	Shen et al. (2012)
Pseudomonas putida HI47	Sludge	PAM	Aerobic		Gel filtration chromatography; FTIR	–	Yu et al. (2015)
Pseudomonas stutzeri	Wastewater	Acrylamide	Aerobic	CO_2	Optical density	NA	Wang and Lee (2001)
Geobacillus thermoglucosidasius AUT-01	Soil near hot spring	Acrylamide	Aerobic	Acrylic acid/ CO_2	Optical density	NA	Cha and Chambliss (2011)
Rhodococcus rhodochrous NHB-2	Soil	Acetamide, propionamide, acrylamide	Aerobic/30 °C	Smaller molecules	HPLC	75	Chand et al. (2004)
Variovorax boronicumulans CGMCC 4969	Soil	Acrylamide	Aerobic	Acrylic acid	Optical density; spectrophotometry	100	Liu et al. (2013)

concentration, where large accumulations of the metabolic intermediate such as acrylic acid may adversely affect microbial activity. Strains of *Pseudomonas* sp., *Xanthomonas maltophilia*, *Pseudomonas aeruginosa*, *Rhodococcus* sp., and *Enterobacter aerogenes* have shown promising results in AA degradation under certain physiological conditions (Nawaz et al. 1993; Prabu and Thatheyus 2007; Nawaz et al. 1998; Thanyacharoen et al. 2012). PAM degradation pathways have been extensively studied in bacteria; however, the complexity of the eukaryotic genome has made it less approachable to study fungal counterparts.

20.3.4 Methods of Detection

Bacteria have dominated the PAM biodegradation scenario for more than two decades. The most common protocol for the determination of degradability potential of a particular microbial strain is the turbidometry method, which utilizes turbidity as the directly proportional parameter for microbial growth (Mauerhofer et al. 2018). Spectrophotometers are generally utilized for the study of bacterial growth based on Beer Lambert's principle. The absorbance of the cell suspension placed in a transparent cuvette is measured as optical density (OD), which is directly proportional to the biomass in the cell suspension in a given range for the cell type. However, the inability to provide an absolute count of living cells or to distinguish from dead cells proves to be a major drawback of the turbidometry method (Duedu and French 2017). Another technique that is reliably used in biodegradation studies is FTIR spectroscopy as it can predict the molecular structure of a compound. Every molecule has a distinct IR pattern as different wavelengths of IR light are absorbed by various chemical bonds which can be distinctly measured (Grabska et al. 2019). Hence, a polymer post breakdown releases monomer along with various molecules which have distinct yet different IR peaks as compared to its parent molecule. In order to quantify the PAM degradation, analytical methods are most widely employed to quantify PAM degradation and to also identify specific functional groups: ketone, aldehyde, and carboxylic groups (Xiong et al. 2018). Due to its polar nature and small size, acrylamide quantification has been proven to be tricky.

Estimation of acrylamide content by gas chromatography-mass spectrometry (GC-MS) method requires derivatization (bromination) step of the samples with an objective to eliminate broadening of maximum peaks with an objective to enrich the identification of ion fragments. The highly soluble nature of acrylamide hinders its extraction from liquid samples. For GC-MS sample preparation, the water content in the extract is removed prior to injection in the instrument to avoid high expansion of water at higher temperatures as it possesses the capability to damage the column over time (Falaki 2019). Usually, sodium chloride or magnesium sulfate is added to the extract with the objective of salting out, allowing AA/PAM to partition into the acetonitrile layer due to the formation of a density gradient, whereas liquid chromatography-mass spectrometry (LC-MS) proves to be a simpler process that completely eliminates the process of derivatization, making it less time-consuming and easier for AA estimation (Eberhart et al. 2005). For water samples,

high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry is preferred because of the sensitivity and selectivity of the technique (Holcapek et al. 2008). However, for all the chromatographic estimation techniques, solid-phase extraction (SPE) is preferred over liquid-liquid extraction as it is simpler and less time-consuming.

20.4 Polyaromatic Hydrocarbons as a Pollutant

USEPA (United States Environmental Protection Agency), OECD (Organization for Economic Co-operation and Development), and many other countries including India have designated petroleum hydrocarbons as hazardous wastes (Ministry of Environment and Forest, Government of India, 2000). In a wider range, petroleum hydrocarbons are categorized into aliphatic (mostly *n*-alkanes, branched alkanes, and cycloalkanes) and aromatic hydrocarbons including monoaromatics (benzene, toluene, ethylbenzene, and xylenes) and polycyclic aromatics (polycyclic aromatic hydrocarbons, i.e., PAHs such as naphthalene, phenanthrene, anthracene, and pyrene) and asphaltics (Nzila 2018). PAHs are a subclass of a set of organic compounds termed polycyclic organic matters (POM) and found ubiquitously in the environment (Choi et al. 2010; Prakash et al. 2011). They are formed due to incomplete combustion, drying, roasting, and/or smoking of organic materials. These PAHs deposit from the air on the surface of plants, water bodies, and on the soil system (Zelinkova and Wenzl 2015). As semi-volatile compounds, PAHs can be transported over long distances in the atmosphere. The solubility and the bioavailability of these hydrocarbons determine the toxic nature of petroleum hydrocarbons and thus its microbial uptake rate and biodegradation (Xu et al. 2018). The term "PAH" refers to compounds comprising of carbon and hydrogen atoms only. PAHs are aromatic hydrocarbons with two or more fused benzene rings bonded in the linear, cluster, or angular arrangements (Yu 2002). They occur as colorless, white/pale yellow solids with low solubilities in water, high melting, and boiling points. With an increase in molecular weight, their solubility in water decreases, and vapor pressure decreases while hydrophobicity/lipophilicity, melting, and boiling points increase (Achten and Andersson 2015). Based upon the origin, PAHs can be categorized into two groups: petrogenic and pyrogenic. Petrogenic PAHs are mainly derived from fossil fuels such as coal, crude oil, and refined products, whereas pyrogenic PAHs are formed as a result of incomplete combustion of fossil fuels, forest fires, and tobacco smoke (Patel et al. 2020). PAHs are ubiquitously found in the environment and are mainly produced by two sources: natural and anthropogenic. Natural sources include biogenic and geochemical sources. Biogenic aromatic compounds include aromatic amino acids, lignin compounds, and their derivatives through biotransformation (Ghosal et al. 2016). PAHs may accumulate in a high concentration in the environment near coal gasification sites and oil distillation plants (Seo et al. 2009). Fossil fuels such as natural crude oil and coal contain significant amounts of PAHs (Perera 2017). In addition, PAHs are also produced during geochemical processes such as pyrolysis, which involves the exposure of

sediments to high temperatures during sediment diagenesis (Ghosal et al. 2016; Singh and Tiwary 2016).

Numerous remediation technologies have been developed for the reduction of hydrocarbon contamination in soil and sediment including both physicochemical and biological methods (Azubuike et al. 2016). PAHs released into the environment can be removed/degraded through many processes, including volatilization, photo-oxidation, chemical oxidation, adsorption, thermal decomposition, bioaccumulation, biodegradation, phytodegradation, or through an integrated approach (Shemer and Linden 2007). Biological methods offer a sustainable alternative to other treatment methods due to their low environmental impact, low costs, and high capability to degrade a wide variety of organic contaminants (Ojuederie and Babalola 2017). However, the leading process used for the successful removal and elimination of PAHs from the environment is microbial transformation and degradation. The basic principle of any remediation technology is to remove or detoxify a contaminant from a given environment. Most of the field trials are inclined to monitor the treatment by the extent of removal/transformation of the parent contaminant; however, the possibility of production of more toxic degradation metabolites is not considered (Koshlaf and Ball 2017). Nevertheless, it is very necessary to ensure that the pollutant/contaminant is properly detoxified into nontoxic end products at the completion of the treatment. It is well known that PAH has mutagenic, teratogenic, and carcinogenic properties, and due to this, these compounds have gained importance in toxicological studies (Ewa and Danuta 2017). In the last few decades, there has been increasing concern over public health due to the release of petroleum hydrocarbons and other pollutants in the environment as a result of anthropogenic activities and natural processes (Manisalidis et al. 2020). Presently, India and many other countries all over the world are facing severe problems due to the persistent nature of these pollutants (Hussain et al. 2018). Soil and water contain measurable quantities of PAHs, mostly from leaching, airborne fallout, accidental oil spills, industrial effluents, and production of primary aluminum, coke, rubber tires, and petrochemicals (Srogi 2007). In India, major sources (90%) of PAHs contamination could be biofuel consumption because the country depends on firewood, straw, animal dung, and petroleum for domestic energy, despite the fact that PAHs emissions from petroleum combustion were much lower than biomass burning (Shen et al. 2013). The major cause of PAHs pollution is mainly through anthropogenic activities, particularly fuel combustion, automobiles, coal liquefaction, coke production, spillage of petroleum products, and waste incineration (Ghosal et al. 2016). PAHs are also formed during the incomplete combustion of organic materials such as coal, diesel, fat, wood, and vegetation (Srogi 2007). Airborne PAHs are the main route for their transport over long distances, whereas tobacco cigarette smoke and burnt foods are a significant source of PAH exposure to both smokers and nonsmokers (Diggs et al. 2011). There are many PAHs used for commercial purposes. They are mainly used as intermediaries in agricultural products, thermosetting plastics, pharmaceuticals, photographic products, lubricating materials, and other chemical industries for the manufacture of resins, pigments, dyes, plastics, agrochemicals, electronics, diluent for wood preservatives, and pharmaceuticals. In

addition to roofing tar, asphalt used for the construction of roads contains many PAHs (Nasher et al. 2013).

20.4.1 Toxicity of PAHs

Generally, the higher the molecular weight of the PAHs, the higher the hydrophobicity, toxicity, and environmental persistence, and the lower the bioavailability of those PAHs (Ghosal et al. 2016). The greater the number of the benzene ring in the structure of PAH, the greater is the toxicity and lesser is degradability (Seo et al. 2009). Association with other pollutants such as heavy metals and hydrocarbons increases the toxicity and persistence of the PAHs in different forms in the environment. PAHs toxicity increases several orders of magnitude by the exposure of the ultraviolet portion of sunlight (Shemer and Linden 2007). PAHs contamination poses adverse effect on the soil microflora and fauna and also reduces the fertility of the soil (Ahmad et al. 2018). These pollutants cause deleterious effects on micro- as well as macroorganisms, including plants and humans and other life forms. Hydrocarbon contaminants such as PAHs are toxic for living organisms as they accumulate in the lipid bilayer of cell membranes and thus disturb the fluidity of membranes (Murínová and Dercová 2014). The severity of PAH's toxic effects is dependent on the parameters such as mode of exposure, duration of exposure, and dose of exposure (Majewski et al. 2018). Organic compounds mainly composed PAHs are one of toxic environmental pollutants. Excessive exposure to PAHs often results in lung cancer as PAH induces various metabolic pathways consisting of both phase I metabolic enzymes such as cytochrome P450 (CYP) monooxygenases, i.e., CYP1A1/2 and 1B1, and phase II enzymes such as glutathione S-transferases, UDP glucuronyl transferases, NADPH quinone oxidoreductases (NQOs), aldo-keto reductases (AKRs), and epoxide hydrolases (EHs), via the aryl hydrocarbon receptor (AhR)-dependent and independent pathways resulting in the formation of diol epoxides, capable of reacting with DNA to produce carcinogenic adducts (Moorthy et al. 2015). PAH health hazards include carcinogenesis, localized skin effects, pulmonary and respiratory problems, genetic reproductive and developmental effects in fetus, and behavioral and neurotoxic anomalies. Recent studies have also correlated PAHs in the air with asthma in children living in developed countries (Kim et al. 2017).

20.4.2 Degradation Pathways of PAH

20.4.2.1 In Bacteria

Bacterial degradation of some PAHs such as naphthalene, phenanthrene, and anthracene has been well studied. Naphthalene (two-ring PAH) is the simplest and soluble PAH, and it is comparatively easy to isolate microorganisms with the ability to utilize naphthalene as the sole source of energy (Ghosal et al. 2016). These microorganisms metabolize naphthalene or other PAHs by oxygenation of the

aromatic ring to form a dihydroxy aromatic intermediate (diol/two alcohol groups) (Seo et al. 2009). In the naphthalene biodegradation pathway, the first enzyme is naphthalene dioxygenase which attacks the aromatic ring to form cis-naphthalene dihydrodiol (dihydroxy-1,2-dihydronaphthalene) (Seo et al. 2009). Consequently, the enzyme cis-dihydrodiol dehydrogenase converts cis-naphthalene dihydrodiol into 1,2-dihydroxynaphthalene (Costa et al. 2017). This dehydrogenated product is eventually metabolized via 2-hydroxy-2H-chromene-2-carboxylic acid, cis-o-hydroxybenzal pyruvate, and 2-hydroxy-benzaldehyde into intermediate metabolite, i.e., salicylate (Costa et al. 2017). Moreover, the metabolite 1,2-dihydroxynaphthalene is converted into 1,2-naphthaquinone by a nonenzymatic process (Swetha and Phale 2005). A report from Costa et al. (2019) states the conversion of 1,4-naphthaquinone into benzoic acid and finally into aromatic ring cleaved intermediate, i.e., catechol, where the product salicylate is usually converted into catechol by decarboxylation, and this catechol is further metabolized in ortho and meta pathways, and salicylate is further hydroxylated into gentisate by the enzyme salicylate 5-hydroxylase. Phenanthrene (three-ring PAH) degradation by bacteria is generally initiated by the enzyme dioxygenase to yield cis-3,4-dihydroxy-3,4-dihydrophenanthrene, which is further metabolized into 3,4-dihydroxyphenanthrene by the enzyme dehydrogenase (Seo et al. 2009). This product (3,4-dihydroxyphenanthrene) is later metabolized into 1-hydroxy-2-naphthoic acid, 1,2-dihydroxynaphthalene, and ultimately into salicylic acid/salicylate (Wang et al. 2018). Phenanthrene, a three-ring PAH, is degraded by the formation of 1-hydroxy-2-naphthoic acid, 1-naphthol, and salicylic acid in the bacterial species *Brevibacterium* sp. HL4, while *Pseudomonas* sp. DLC-P11 metabolized phenanthrene via 1-hydroxy-2-naphthoic acid, 1-naphthol, and o-phthalic acid (Gao et al. 2013). Pyrene, a four-ring PAH, can be mineralized by many bacterial species including *Mycobacterium* through ring oxidation and ring fission process (Kanaly and Harayama 2000). The main metabolic products of the ring oxidation process are cis-4,5-pyrenedihydrodiol, trans-4,5-pyrenedihydrodiol, and pyrenol, and on the other hand during the ring fission process, 4-hydroxyperinaphthenone, 4-phenontheroic acid, phthalic acid, and cinnamic acid are formed. Presence of other metabolites such as 4-cis- and trans-4,5-dihydrodiols proposes multiple pathways for the initial oxidative attack of pyrene structure (degrade several PAHs including phenanthrene, pyrene, and benzo(a)pyrene) (Moody et al. 2004).

20.4.2.2 In Fungi

Years of research has revealed that the first step in the PAH metabolism by non-ligninolytic fungi is the oxidation of the aromatic ring to an arene oxide by a cytochrome P450 monooxygenase enzyme catalyzed reaction, a step contrasting to the mechanism of action of dioxygenase enzymes which leads to the formation of cis-dihydrodiols from the aromatic ring (Ghosal et al. 2016). This step is followed by the hydration of cis-dihydrodiols to trans-dihydrodiols via an epoxide-hydrolase catalyzed reaction. Through nonenzymatic rearrangement the phenol derivatives of the arene oxides act as substrates for subsequent sulfation or methylation or

conjugation with glucose, xylose, or glucuronic acid (Wang et al. 2018). However, these PAH conjugates are considered less toxic and more soluble as compared to their respective parent compounds. Ligninolytic fungi produce three different types of extracellular enzymes, i.e., lignin peroxidase (LP), manganese peroxidase (MnP), and laccases, which are responsible for the oxidation of lignin present in wood (Agrawal and Verma 2020b; Agrawal et al. 2019; Bhardwaj et al. 2020; Verma and Madamwar 2002) and other organic matter via a nonspecific radical-based reaction with an ability to diffuse into the soil/sediment matrix and can potentially oxidize PAHs with low bioavailability (Agrawal et al. 2018; Agrawal and Verma 2020c; Kumar and Chandra 2020). The ligninolytic fungi oxidize PAHs by generating hydroxyl free radicals leading to the formation of quinone derivatives of PAHs (Pozdnyakova 2012). However, researchers have suggested the cumulative involvement of cytochrome P450 monooxygenases, ligninolytic enzymes, and epoxide hydrolases in the degradation of PAHs resulting in the complete mineralization of the parent compound (Cerniglia and Sutherland 2010).

20.4.2.3 Anaerobic Conditions

Aerobic environments such as contaminated soils, marine sediments, and groundwater occasionally can develop anaerobic zones resulting from the depletion of molecular oxygen due to the stimulation of the microbial consortia present in situ in response to the organic contaminant such as PAHs (Hidalgo et al. 2020). These anaerobic zones are found to be rich in nitrate, sulfate, iron, manganese, and carbon dioxide that can act as electron acceptor in the breakdown of organic compounds into smaller final constituents such as carbon dioxide and methane (Orcutt et al. 2011; Pester et al. 2012). Primarily, anaerobic biodegradation is enforced when the degree of contamination is very high, limiting oxygen flow due to organic matter pore saturation or clogging of aggregates (Yoshikawa et al. 2017). Considerably, this innovation serves as a promising remediation measure for accidental oil spills and remediation of water-logged soil, for example, paddy fields and swamps. Furthermore, anaerobic biodegradation is foreseen to supplant oxygen-consuming biodegradation since an enormous air circulation zone is not important to lessen complete remediation cost. Since the cycle is not oxygen subordinate, anaerobic bioremediation may be applied for the treatment of underground soil and groundwater (Azubuike et al. 2016) (Fig 20.2). The overall success and speed of bioremediation applications are largely dependent on different factors such as soil characteristics, nutrient availability, oxygen, and bioavailability of the contaminants that vary from site to site (Ghosal et al. 2016). The most frequent limiting nutrients for PAHs biodegradation are nitrogen (N), phosphorus (P), and potassium (K) as microorganisms require these minerals for their cellular metabolism and growth (Ghosal et al. 2016). Available nutrients are rapidly depleted during microbial metabolism in contaminated sites, where organic carbon levels are often high due to the nature of the pollutant (Das and Chandran 2011; Azubuike et al. 2016).

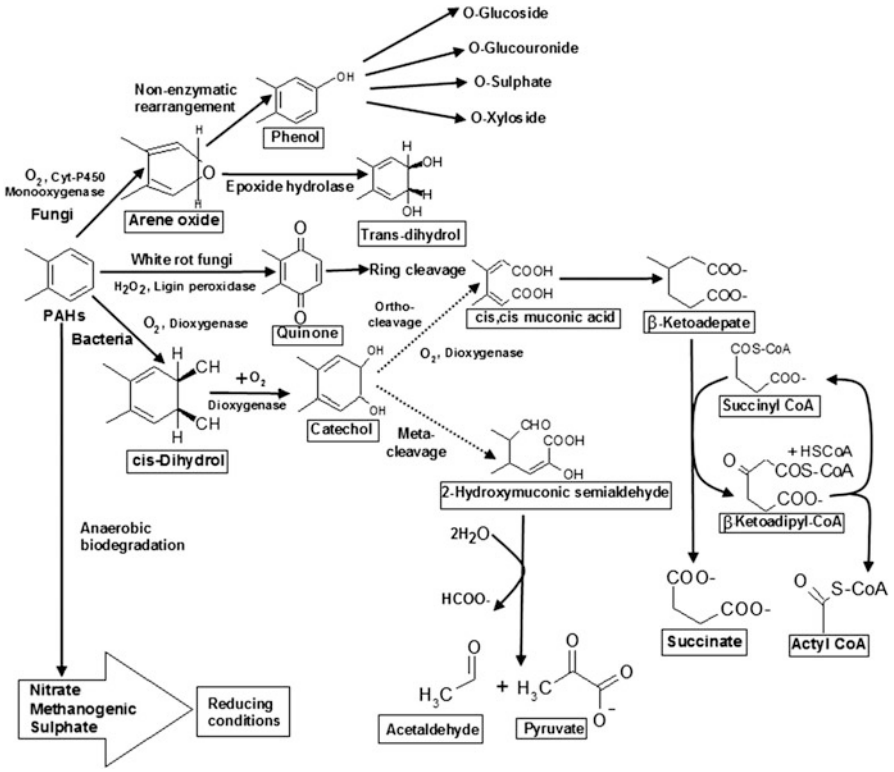

Fig. 20.2 Current knowledge of microbial pathways for microbial remediation of PAH (Cerniglia 1992)

20.4.3 Approaches for Bioremediation of PAH

Multiple studies have revealed that the degradation potential of bacteria is above fungi and yeast as they can sustain through molecular changes leading to the production of catalytic enzymes suitable for the respective substrate (Janusz et al. 2017). Owing to it's smaller size and ability to form biofilms, bacteria have relatively increased active surface area for efficient biodegradation of PAHs as compared to larger cell size of fungi and algae which make them susceptible to environmental conditions like availability of nutrients, sunlight, moisture, etc. restricting their efficacy towards potential PAH degradation (Sakshi and Haritash 2020). Numerous remediation technologies have been developed for the reduction of hydrocarbon contamination in soil and sediment including both physicochemical and biological methods (Xu et al. 2018). Bacterial remediation of PAH has been reported by organisms belonging to various genera such as *Pseudomonas*, *Alcanivorax*,

Microbulbifer, *Sphingomonas*, *Micrococcus*, *Cellulomonas*, *Dietzia*, *Gordonia*, *Marinobacter*, *Mycobacterium*, *Haemophilus*, *Rhodococcus*, *Paenibacillus*, *Bacillus*, *Aeromonas*, *Burkholderia*, *Xanthomonas*, *Micrococcus*, *Arthrobacter*, *Acinetobacter*, *Corynebacterium*, and *Enterobacter* (Nzila and Musa 2020) (Table 20.3). The rate of degradation is mainly dependent on the molecular weight and solubility of the PAHs (Nanca et al. 2018). Metabolism or co-metabolism is the main process involved in PAHs degradation by the bacteria (Ghosal et al. 2016). PAHs can be degraded under both aerobic and anaerobic conditions but most attention has been paid to the aerobic metabolism of PAHs and the enzymatic and genetic regulation involved in the degradation process (Brzeszcz and Kaszycki 2018). These species degrade PAH compounds by a cumulative process where the PAH is used as the substrate for meeting the energy requirements for growth, leading to mineralization of the compound (Jinadasa et al. 2020). The presence of molecular oxygen is required for initiating the enzymatic attack to PAH rings in aerobic biodegradation by bacteria or fungi (Ghosal et al. 2016). Although oxygen is not the only electron acceptor but it is the most common one in bacterial respiration and via oxygenation reactions, it activates the substrates. The initial step in the aerobic catabolism of a PAH molecule by most of the bacteria generally includes the oxidation of the benzene ring by mono- or dioxygenase enzymes (Seo et al. 2009). This reaction leads to the conversion of the aromatic compounds into dihydroxy aromatic intermediates (dihydrodiols), the latter getting dehydrogenated to form dihydroxylated compounds (Díaz et al. 2001; Pérez-Pantoja et al. 2010). This is followed by an ortho-cleavage pathway (in which the ring is cleaved between the two carbon atoms with hydroxyl groups) or a meta-cleavage pathway (in which the ring cleaves between adjacent carbon atoms with or without a hydroxyl group) depending on the intradiol or extradiol ring cleaving (Haddock 2010). Most aromatic compounds, including polyaromatics, are known to be metabolized to some common ring cleaved intermediates, such as protocatechuates and catechols (Rajkumari et al. 2018). Catechol is further oxidized through the two ring cleavage pathways, ortho- and meta-cleavage pathways, by catechol 1,2-dioxygenase (C12O) and catechol 2,3-catechol-dioxygenase (C23O), respectively (Mishra and Singh 2014). Products of ortho- and meta-cleavage pathways are then subjected to subsequent central pathways. These central intermediates are further converted to tricarboxylic acid cycle (TCA) intermediates (Pérez-Pantoja et al. 2016). Due to their ability to endure extreme environmental conditions, fungi possess extracellular enzymes that are effective such as lignin peroxidase, manganese-dependent peroxidases, laccases, cytochrome P450 monooxygenase, and epoxide hydrolase that efficiently oxidize PAHs, further enhancing their proficiency (Ghosal et al. 2016; Janusz et al. 2017). Most of the reported fungal strains cannot use PAH as the sole carbon source for meeting metabolic requirements (Boonchan et al. 2000). The two types of metabolic degradation pathways are mediated by the non-ligninolytic and ligninolytic fungi (a.k.a. white-rot fungi). The majority of fungi to have shown PAH degradation capabilities are non-ligninolytic and, thus, have no need for the lignin peroxidase

Table 20.3 . Bacterial species involved in the degradation of PAHs and their degradation products

PAHs	Bacteria	Degradation metabolites	References
Naphthalene	*Streptomyces* sp.	Diphenylethylene, 3-nitrophthalic acid, phthalic acid, monocyclohexyl, phenol, 2,4 bis (1,1-dimethylethyl), benzene, 1-methyl-2-(1 methylethyl)	Ferradji et al. (2014)
	Micrococcus sp., *Bacillus* sp., *Staphylococcus* sp., *Pseudomonas* sp.	Catechol, 1,8-dicarboxylic acid, 1-naphthoic acid, and salicylic acid	Pawar (2015)
Acenaphthylene	*Acinetobacter* sp. *Rhizobium* sp. *Sphingomonas* sp.	Acenaphthenequinone, naphthalene-1,8-dicarboxylic acid, gentisic acid, 1-naphthoic acid, salicylic acid, acenaphthene cis-1,2-dihydrodiol	Ghosal et al. (2016); Poonthrigpun et al. (2006); Pinyakong et al. (2003)
Phenanthrene	*Stenotrophomonas maltophilia*	1,2-dihydroxyphenanthrene, 3,4-dihydroxyphenanthrene, cis-9,10-hidroxyphenanthrene, 5,6-benzocoumarin, 7,8-benzocoumarin, methyl trans-4-(1-methoxy-2-naphthyl)-2-oxobut-3-enoate, 2-carboxyvinyl-1-naphthoate, 2–20-diphenic acid, 2-hydroxy-1-naphthaldehyde, 1-hydroxy-2-naphthaldehyde, 2-hydroxy-1-naphthoic acid, 1-hydroxy-2-naphthoic acid, naphthalene-1,2-dicarboxylic acid, 1,4-dihydroxy-2-naphthoic acid methyl ester, naphthalene-1,2-diol,2-carboxybenzalpyruvate, coumarin, 2-carboxycinnamic acid, 2-formylbenzoic acid, salicylic acid, phthalic acid, and protocatechuic acid	Gao et al. (2013)
Anthracene	*Martelella* sp.	3-hydroxy-2-naphthoic acid, 1-hydroxy-2-naphthoic acid, 2-hydroxy-1-naphthoic acid, 3-hydroxy-2-naphthoic acid, 2,3-dihydroxynaphthalene, 2,3-dihydroxyanthracene, 9,10-anthraquinone, 6,7-benzocoumarin, gentisate acid	Cui et al. (2008)

enzymes that are produced by the ligninolytic fungi. Instead, non-ligninolytic fungi produce cytochrome P450 monooxygenase enzymes that can oxidize PAHs to form arene oxide ultimately leading to the formation of conjugative phenols of xylose, gluconic acid, and glucose through nonenzymatic rearrangement. Ligninolytic fungi are known to produce extracellular enzymes such as lignin peroxidase, manganese peroxidase, and laccases responsible for the degradation of wood lignin. These enzymes catalyze the cleavage of aromatic compounds to water-soluble polar products that are subsequently utilized for fungal metabolism. Some of the notable PAH-degrading fungal strains are *Phanerochaete*, *Pleurotus*, *Trametes*, *Bjerkandera*, *Chrysosporium*, *Cunninghamella*, *Coriolopsis*, *Aspergillus*, *Penicillium*, *Trichoderma*, *Mucor*, and *Cladosporium* (Sakshi and Haritash 2020). However, factors such as growth, inadequate biomass, etc., are some of the drawbacks that have probably contributed to the lesser understanding of fungal pathways responsible for PAH mycoremediation.

20.4.4 Methods of Detection

For the detection of PAHs, the methods have been grouped into three categories: immunoassay, spectrometric, and chromatographic. Immunoassay methods because their lowered precision, accuracy, and affinity for many aromatic compounds are mostly used for field screening of soil and water samples for PAH contamination (Olagoke et al. 2017). Among the spectrometries, UV methods (absorption and fluorescence) are considered to be sensitive and selective toward aromatic PAHs. Similarly, the IR spectrometric method is considered to be fast and affordable, requiring a mandatory cleanup step after extraction before analytical determination of the sample. Among the chromatographic methods, GC is extensively used due to the ease of handling thermally labile, semi-volatile, less volatile, or nonvolatile PAHs compounds.

20.5 Advantages and Disadvantages of Bioremediation

Being a natural process, bioremediation does not produce any secondary metabolites/compounds during the metabolism, ensuring complete removal of toxicants or altering their state to a much less toxic form. Because of the usage of cost-effective techniques, it is any time preferable to clean up contaminated sites, without disrupting the normal flora and fauna. Though minuscule, the main restraint factor for bioremediation techniques is it not being an expeditious technology. Sometimes it is not advisable to adopt, since the product anticipated after decontamination can be more toxic than the parent compound. However, the biggest challenge lies in the transition of pilot-scale studies onto field studies, as the process is mostly dependent on the onsite factors such as indigenous microbial population and level of nutrients and contaminants, apart from environmental conditions (Pratush et al. 2018).

20.6 Conclusion

Heavy metals, PAHs, acrylamide, and polyacrylamide discharged into the environment are assiduous due to their toxicity propounding an intense hazard to organisms exposed to high levels of such pollutants. Bioremediation is progressively being endorsed for the management and restoration of contaminated sites because of its advantages over conventional chemical and physical methods, which are not cost-effective and specific, and produce more toxic intermediates. Many new technologies are being implied by researchers to stun the detriments of bioremediation. However, the search for potent technology is always sought after, which can aim for new mechanisms and genetically modified organisms to enhance bioremediation potential.

Competing Interests All the authors declare that they have no competing interests.

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Microbes and Their Application in the Food and Agriculture Industry

21

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Abstract

As the world population is growing exponentially, there is enormous pressure on land resources to ensure food security. The exploitation of forest areas for agricultural purposes can have serious environmental consequences. Microbes, which are an integral part of the soil environment, play an important role in increasing the yield of crops. Their activities play a central role in the soil environment, influencing soil properties and thereby affecting the quality and quantity of agricultural products. They have found their application in biocontrol of pests and insects, increasing soil fertility and promoting plant growth. In the food industry, they are important in the processing and preservation of food. In addition, the protein-rich microbial cells are cultivated as food supplements. Microbes are thus important for the improvement and sustainability of agriculture and food production.

Keywords

Microorganism · Biofertilizers · Biocontrol · Food processing · Preservation

Abbreviations

ACC	1-Aminocyclopropane-1-arboxylic acid
CAZymes	Carbohydrate active enzymes
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_21

ISFM	Integrated Soil Fertility Management
PABA	Para-aminobenzoic acid
PGPM	Plant growth-promoting microorganisms
PGPR	Plant growth-promoting rhizobacteria
PSM	Phosphate-solubilizing microorganisms
SAR	Systemic acquired resistance
WHO	World Health Organization

21.1 Introduction

Global agriculture and the food sector are under constant pressure to provide enough food for the exponentially growing world population. Limited land resources make use of fertilizers, biological control agents, and tillage practices the obvious alternatives (Carvalho 2006). However, the use of chemical-based fertilizers and pesticides is detrimental to the environment in due course and has food safety issues (Taylor et al. 2002). The use of various chemical preservatives and rations to extend the shelf life of food and thus improve food safety are also completely safe for consumption (Macfarlane 2002).

According to the 2030 Agenda adopted by the United Nations on September 25, 2015, the second Sustainable Development Goal focuses on ending hunger and providing food security by promoting sustainable agriculture which is targeted to be achieved by 2030 (Assembly 2015). Microorganisms can play a crucial role in achieving these goals as they are actively involved in various biological processes and sustaining life on earth (Akinsemolu 2018). By exploring microbial diversity for their various roles in nature, these organisms can be used to increase agricultural productivity and application in the food industry without any negative effects as in the case of various chemicals (Pereg and McMillan 2015).

The microbes have the ability to improve soil health by mineralizing nutrients and increasing soil fertility, biocontrol, and can also help in promoting plant growth without any negative impact on the environment (Johansson et al. 2004). Improving food processing, storage, and preservation techniques is as important for food security as increasing agricultural productivity (Akinsemolu 2018). Again, the microorganisms can be used to increase the shelf life of various food products as well as to convert food waste into an edible form using fermentation technology (Nout and Kiers 2005; Ross et al. 2002). In addition, the various fermentation products are important commercial products in the food industry (Marshall and Mejia 2011). The microorganisms can be used as probiotics to increase metabolism as well as to produce various metabolites that can be used in food processing and preservation (Ross et al. 2002).

This chapter considers the function assumed by the microbes in the agriculture and food industry and their application in developing a sustainable system to combat the rising population and global hunger.

21.2 Role of Microbes in Agriculture and Increasing Crop Yields

Sustainable agriculture is a concept where high crop yields are achieved without compromising normal soil properties such as fertility, water content, etc. However, such an ideal condition is practically not possible with limited land resources on which extensive agriculture is practiced. Various strategies are being developed in the field of agriculture to enhance crop productivity and nutritional value. The methods currently used are dependent on the large-scale use of chemical-based fertilizers and pesticides. However, the harmful effects of these chemicals on the natural environment and human well-being have necessitated a reevaluation and search for environmentally friendly alternatives for these compounds. To increase productivity from limited resources without affecting the quality of crops or soil, microorganisms can be used in agriculture as they play an important role in soil ecology. The microorganisms having a positive impact on plant growth are now successfully used as green technology in this sustainable agriculture process (Singh et al. 2017). The plants form an interaction with the huge microbial load that resides in their rhizosphere, and this interaction of microbes and the plants can be beneficial or detrimental to the plants (Glick 2018). These beneficial roles of microbes also called plant growth-promoting microorganisms (PGPM), especially rhizobacteria (PGPR) that dwell in the rhizosphere (Fig. 21.1.), are being studied and applied in the field of agriculture for the following purposes.

21.2.1 Microbes as Biofertilizers and in Nutrient Cycling

A major problem for the productivity of agricultural land is the low availability of nutrients. Since plants require various nutrients in appropriate proportions for their optimum growth, various strategies are adopted to increase the fertility of the soil. Integrated soil fertility management (ISFM) is one such strategy in which biological fixation of atmospheric nitrogen, conservation of natural resources, and increasing

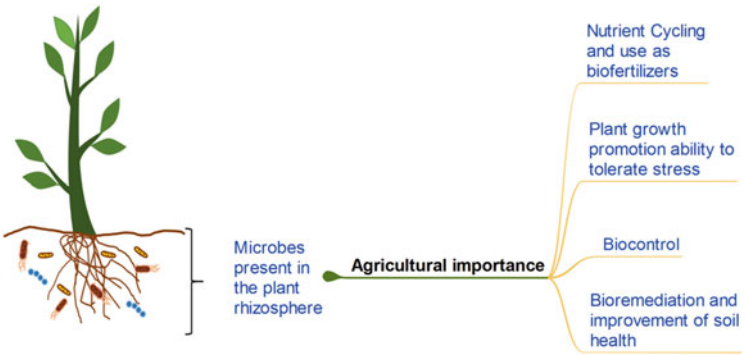

Fig. 21.1. Microbes in the soil and their significance in agricultural practices

the competence of inputs are the aspects used to increase soil fertility (Vlek and Vielhauer 1994).

An important step for the management of nutrient content is the use of fertilizers. The utilization of chemical fertilizers to boost agricultural productivity can have negative effects on the soil environment and human health. Therefore, the living cell-based fertilizers or the "biofertilizers" are the alternatives to the chemical fertilizers used in sustainable agriculture due to their environmental and cost-friendly aspects (Mohammadi and Sohrabi 2012). The living organisms that formulate the biofertilizers colonize either the plant interior or the rhizosphere to cause mineralization or fixation of the essential elements to convert them from an inaccessible form to an accessible form that can be used for plant growth (Rokhzadi et al. 2008). These organisms cause bioconversion of various nutrients and minerals to increase their bioavailability, resist pathogenic invasion, and also produce various phytohormones, amino acids, etc. (Malusá and Vassilev 2014). The biofertilizers play a role in increasing the growth rate of plants through various mechanisms. PGPRs are a commonly used biofertilizer that can even secrete various growth-promoting phytohormones that rapidly pass through the cell cycle under the ideal conditions of sufficient light, water, and minerals (Wong et al. 2015). The various groups of microorganisms used as biofertilizers include nitrogen fixers, phosphate solubilizers, and potassium solubilizers (Mohammadi and Sohrabi 2012).

Nitrogen, one of the most essential macronutrients needed for plant development and growth, is inaccessible to plants even though it makes up 78% of atmospheric gasses, making it the primary limiting nutrient for optimal plant growth. Nitrogen-fixing microorganisms, which occur either as a free-living or in a symbiotic relationship with the plant, fix this atmospheric nitrogen into ammonia, which is then assimilated by plants for their nutrient needs (Tairo and Ndakidemi 2013). Bacterial species belonging to *Rhizobium* such as *Rhizobium leguminosarum*, *Rhizobium trifolii*, *Rhizobium phaseoli*, and *Rhizobium meliloti* as well as *Frankia* and *Azolla* carry out biological nitrogen fixation in a symbiotic relationship with legumes, while *Cyanobacteria* and *Azotobacter* sp. and *Azospirillium* sp. (*Azotobacter chroococchum*, *Gluconacetobacter diazotrophicus*, *Azospirillum lipoferum*, *Azotobacter vinelandii*) are found to be free-living (Gupta 2004; Singh et al. 2017). The cyanobacteria or blue-green algae not only contribute to nitrogen fixation but also produce various plant hormones such as auxins, indoleacetic acid, and gibberellic acid, and studies show their importance in improving rice productivity (Watanabe and Roger 1984).

Soil phosphorus content is usually in an insoluble form such as rock phosphate, which makes it the second limiting nutrient for plant growth. Thus the other important group of microorganisms are the phosphate-solubilizing microorganisms (PSM) which convert the inorganic insoluble form of phosphorus into soluble forms (HPO_4^{2-} and H_2PO_4) and make it available to plants through various processes such as the production of organic acid (gluconic acid, glyoxylic acid, oxalic acid, acetic acid, maleic acid, citric acid, tartaric acid, lactic acid) (Kim et al. 1997), ion exchange, and chelation. This property of these organisms is of additional benefit in their use as biofertilizers to make the other insoluble components of the fertilizer

Table 21.1. Commercially available microorganism-based biofertilizers

Biofertilizer brand	Microorganism	Company
Jumpstart®	*Penicillium bilaiae*	Novozyme
Quickroots®	*Bacillus amyloliquefaciens* and *Trichoderma virens*	Novozyme
Optimize®	*Bradyrhizobium japonicum*	Novozyme
Lalrise Max Wp	Endomycorrhizal spores	Lallemand Plant Care
Bysi-N Liquid	*Bradyrhizobium japonicum*	Lallemand Plant Care
Lalfix Start Spherical Pea & Lentil	*Rhizobium leguminosarum* biovar *viciae and Bacillus velezensis*	Lallemand Plant Care
Bluen	*Methylobacterium symbioticum*	Symborg
Rizoliq Peanut in Furrow	*Bradyrhizobium* sp.	Rizobacter Argentina S.A.
Ribol	*Rhizobium leguminosarum* bv *trifolii*	Rizobacter Argentina S.A.
Jingaiguibao	*Bacillus subtilis*, *Paenibacillus kribbensis*, *Bacillus megaterium*	Kiwa Bio-Tech Products Group Corporation

also accessible (Banerjee et al. 2010; Chang and Yang 2009). The organisms belonging to bacterial and fungal genera such as *Bacillus*, *Penicillium*, *Aspergillus*, *Rhizobium*, *Pseudomonas*, and *Enterobacter* are the most efficient phosphate solubilizers (Whitelaw 1999).

The arbuscular mycorrhizal fungi deserve special mention here because this group of fungi forms an association with the plant in which the fungal partner receives the carbon source from the plant while providing the plant with various nutrients from the soil. The mycelium of the fungus penetrates deeper into the soil and helps in efficient nutrient uptake by the plant, although they themselves do not have the ability to solubilize phosphate (Debnath et al. 2019).

The other important microorganisms with potentials as biofertilizers include organic matter decomposers such as *Aspergillus niger*, *Phanerochaete chrysosporium*, *Penicillium*, *Trichurus spiralis*, *Trichoderma viride*, etc., which efficiently degrade organic matter containing recalcitrant biomass such as cellulose and lignin. Potash, sulfur, and zinc are other important nutrients required for plants, and the organisms that solubilize these essential nutrients are also used as biofertilizers to overcome their deficiency in the soil (Debnath et al. 2019). The list of commercially available biofertilizers are provided in Table 21.1.

21.2.2 Soil Health and Bioremediation

The green revolution and extensive use of herbicides, pesticides, and chemical fertilizers have contributed to soil pollution along with other anthropogenic influences (Tilman 1998). Residues of these chemicals lead to contamination of agricultural land, and the resulting effects on crop yield and food safety are of concern (Kopittke et al. 2019). These chemicals are still used for agricultural purposes, although some of them are even considered to have carcinogenic

properties and their accumulation in the soil is of concern for environmental health (Damalas and Eleftherohorinos 2011). There are reports of uptake of these chemicals by crops (Van Dillewijn et al. 2007; Juraske et al. 2011), with an estimated 40% of foods containing trace amounts of pesticides according to FAO-WHO reports (Maksimov et al. 2011). Consumption of these foods can have serious effects on human health due to their high toxicity (Baez-Rogelio et al. 2017). The application of microorganisms with the dual capacity to perform bioremediation of contaminated soil while promoting plant growth can be used in sustainable agricultural practices to combat these problems.

Bioremediation is a biological process in which contaminants are removed from the environment with the help of living organisms that either degrade or convert the contaminant into compounds with low toxicity. Bioremediation of agricultural land involves the use of plants and microorganisms living in the soil. Numerous studies show the potential of the microorganisms in degrading the pollutants (Jabeen et al. 2015; Ramya et al. 2016; Singh et al. 1999). One study reported the isolation of 2,4-D herbicide-degrading *Serratia marcescens* and *Penicillium* sp. This herbicide has been classified by WHO (World Health Organization) as a hormone herbicide with carcinogenic potential and toxicity level II. Thus, the isolated organisms can be effectively used in bioremediation (Silva et al. 2007). Among the different organisms found in soil, bacteria play the main role in chemical degradation as they have the ability to evolve according to the environment. *Acinetobacter*, *Bacillus*, *Alcaligenes*, *Klebsiella*, and *Flavobacterium* are some of the species that have been reported to have the potential to degrade pesticides (Huang et al. 2018).

In addition, the microorganisms also help in phytoremediation by plants, focusing on the PGPRs, whereby these organisms increase the tolerance of the plants while promoting their growth and yield of biomass. Numerous studies have been conducted in which PGPRs were inoculated with toxic resistance and plant growth-promoting properties to increase the efficiency of phytoremediation (Ma et al. 2011). The major contributors to phytostabilization and phytoremediation are the microbes found in the rhizosphere of the soil. *Azotobacter* sp. is among the most commonly used PGPRs, which have also been identified with the ability to degrade various chlorinated pesticides and other toxic compounds (Gurikar et al. 2016). Similarly, a laboratory study was carried out using four PGPR strains of *Bacillus*, i.e., *Bacillus subtilis* GB03, *Bacillus amyloliquefaciens* IN937a, *Bacillus pumilus* SE34, and *Bacillus subtilis* FZB24, using pesticides metribuzin, acibenzolar-S-methyl, napropamide, thiamethoxam, and propamocarb hydrochloride at different concentrations. The experiment demonstrated the ability of these PGPRs to efficiently remove the toxins from the culture media within 72 h (Myresiotis et al. 2012). Thus, the development of a microbial inoculum that acts as a biofertilizer and simultaneously performs bioremediation can increase agricultural yield without negative environmental impact.

21.2.3 Biocontrol

An important aspect of developing a sustainable agricultural system is protecting food crops from pests and pathogens. These pests are an integral part of the agricultural system and have evolved along with their host plants and even show resistance to the various pesticides used in the long run. It has been estimated that global yield losses of major crops, namely, wheat, rice, maize, potato, and soybean, average 21.5%, 30%, 22.5%, 17.2%, and 21.4%, respectively (Savary et al. 2019).

The accumulation of plant exudates in the characteristic form of amino acids and sugars makes the rhizosphere a suitable habitat for the growth of various microorganisms, and these microorganisms residing in the rhizosphere of plants can also help to increase crop yield by exhibiting antagonistic mechanisms toward the pests and pathogens that destroy the plants (Beneduzi et al. 2012). These organisms can do this through different approaches. The plant beneficial organisms can produce various hydrolytic enzymes such as cellulase, proteases, chitinase, and glucanase which can cause the destruction of the cells of these pathogenic fungi, insects, etc. The PGPRs *Serratia marcescens* GPS 5 and *Pseudomonas aeruginosa* GSE 18 produced the CAZymes chitinase along with β-1,3-glucanase, peroxidase, and the enzyme phenylalanine ammonia lyase which significantly reduced the lesion formation by the peanut pathogen *Phaeoisariopsis personata* (Kishore et al. 2005). Another approach would be competitive inhibition of pathogens, whereby these organisms can occupy the niches and nutrients essential for pathogen growth (Kamilova et al. 2005).

These organisms can also produce various secondary metabolites such as antibiotics, bacteriocins, biosurfactants, and siderophores which impede the growth of the pathogens and help in antibiosis to prevent various plant diseases. The endophytic fungus *Acremonium coenophialum* produces alkaloids that protect its host plant by making it unpalatable to the pests (Belesky et al. 1987). The production of siderophores and iron chelation by development makes it inaccessible to pathogens. Thus, this competitive advantage of siderophore-producing PGPRs helps protect plants from various infections (Beneduzi et al. 2012).

These organisms can also induce systemic resistance in the plants to fight the disease. Here, PGPRs provide a similar stimulus to the plant as pathogen-induced systemic acquired resistance (SAR) and help the plant develop resistance to the actual pathogenic infection, although the signaling pathway for these two events is different. These organisms can regulate the level of the plant hormone ethylene in response to an infection via the production of the enzyme ACC deaminase, which acts on 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor molecule of ethylene (Van Loon et al. 1998). Several reports show increased resistance of the plants on treatment with PGPRs (Choudhary and Johri 2009; Ryu et al. 2004). *Klebsiella* sp. MBE02, a halotolerant PGPR, activated the ethylene and jasmonic acid-related plant defense signaling pathway to confer resistance to the peanut plant against *Aspergillus* and other fungal pathogenic infections (Sharma et al. 2019).

21.3 Microbes in Food Production

Microbes not only help to increase soil fertility and crop production but are also used as the food itself or in various food processing technologies to increase the shelf life and the nutritional value of various food products (Fig. 21.2).

21.3.1 Microbes in Food Processing and Preservation

The developing world and the growing population with different socioeconomic and demographic patterns and different food preferences, tastes, and perceptions of different types of food have kept increasing the pressure to develop food preservation methods. The large-scale production of food by various food manufacturers and processors requires proper processing and preservation of food to extend its shelf life without compromising the quality of food such as texture, odor, taste, etc. (Ray 1992). There are various physical and chemical methods of preserving food such as dehydration, vacuum packing, treatment with chemical preservatives, cold storage, etc., but each of these methods has its drawbacks. Moreover, when chemical preservatives are used, there are safety concerns from consumers, which is why the use of bio-based methods is important in food processing (Amit et al. 2017). These synthetic chemical-based food products have been found to have side effects such as gastrointestinal upset and allergic reactions even when used below the threshold concentration as per regulatory guidelines. Therefore, microbes are preferred as effective biopreservatives for their use in various food processing methods to preserve the nutritional value and quality of food (Ng et al. 2019).

The various fermentation approaches used for food preservation require the microorganisms to perform metabolic actions to cause oxidation of carbohydrates. End products of a fermentation process are the various organic acids such as lactic acid and acetic acid, CO_2, and alcohol, which have an inhibitory effect on the growth of the pathogenic and food spoilage-causing organisms. Moreover, the process improves nutritional importance, helps in better digestion and nutrient absorption of food, and can also improve the taste by producing acetaldehyde and diacetyl.

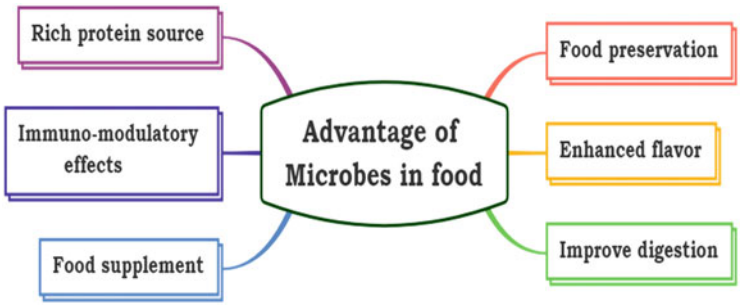

Fig. 21.2. Significance of microbes for their application in food production and consumption

Lactic acid bacteria are the most abundant food organisms that carry out the process of fermentation in most foods (Ross et al. 2002). Other microorganisms, from bacteria to yeasts to molds, are also involved in the fermentation process of various food products, from dairy products to vegetables to meat products (Katz 2001).

The microorganisms also produce bacteriocins, which are proteinaceous toxins produced by the various fermentative bacteria to inhibit other undesirable spoilage or pathogenic microorganisms in the food. The most studied and commercially used bacteriocin is nisin. It is highly specific and shows high efficacy even at very low concentrations. It has been used to extend the shelf life of various dairy products such as cottage and processed cheese and desserts to nondairy products such as liquid eggs. It has been successfully used to prevent spoilage of beer, wine, and various vegetables during their fermentation (Ross et al. 2002). Other bacteriocins include lactins and pediocins, which have also been studied for their use in extending the shelf life of foods such as pasteurized milk but are not yet commercially available (Ross et al. 2002; Simha et al. 2012).

Probiotics, described later in the chapter, are also used as starter cultures for food preservation, and one study showed that the use of microorganisms that act as probiotics, along with the production of bacteriocin, can improve food quality while increasing shelf life when a study was conducted with the probiotic strain *L. acidophilus*. The probiotic organism was able to exhibit antibacterial properties against a variety of food pathogens through the production of bacteriocin (Karthikeyan and Santhosh 2009).

The various metabolites from yeast can also be used as biopreservatives. Flavonoids and phenolic compounds produced by yeast have been shown to have effective antagonistic properties against food pathogens. The yeast *Saccharomyces cerevisiae* produce compounds such as naringenin, phloretic acid, phenylacetaldehyde, and homogentisic acids that exhibit antimicrobial and antioxidant activity with higher efficiency than pure flavonoid, making them a potential candidate for use as biopreservatives (Ng et al. 2019).

21.3.2 Food Supplements

Given the challenges and strain on the agricultural system to meet the needs of the increasing population, microbes are now being used as alternative sources of food. Since livestock and aquaculture are limited by resource requirements, the search for an alternative source of food has led to the use of microbes as a food source. Moreover, the increasing demand for functional foods has greatly contributed to the exploration of the application of various microorganisms, where some of these microbes have long been included in our normal dietary habits, such as edible mushrooms, algae, and cyanobacteria, etc.

21.3.2.1 Edible Mushrooms

Proteins are among the key nutrients required for growth, development, and maintenance whose deficiency can lead to fatty liver and other chronic diseases (Ampong

et al. 2020; Phillips 2017). Since the daily body protein requirement of a healthy adult is 0.80 g/kg/day, and its quality and deficiency having serious implications for health, plant-based sources of protein lack some of the vital amino acids for their use as dietary supplements to meet the globally rising food demand along with those of animal origin, and edible mushrooms are advantageous in their application as a protein source (González et al. 2020).

Mushrooms have always been an integral part of the human diet and are important for their nutritional as well as medicinal values. Edible mushrooms are among the nutritionally rich food sources with the presence of all essential amino acids with high dietary fiber, vitamin, and mineral content, and low levels of calories and fat and have been considered as functional foods. The presence of various bioactive compounds such as essential oils, flavonoids, lectins, ascorbic acids, terpenoids, glycosides, various organic acids, etc., which have immunomodulatory effects such as antifungal, antibacterial, and antidiabetic properties, makes mushrooms a commercial dietary supplement for various diseases, apart from being an important source of protein (Üstün et al. 2018).

The fungi can be grown using simple and readily available lignocellulosic wastes such as straws and coffee grounds as substrates without the need for complex equipment or energy sources. Moreover, the substrates can be used as feed after harvesting, making the process economically feasible and environmentally friendly. Out of over 2000 species, 981 varieties of mushrooms are approved for human consumption and are considered delicacies for their nutritional value and palatability in addition to their medicinal value. Of these, 92 are grown for commercial purposes (Popa-Vecerdea and Oancea 2020).

According to the data provided by the Food and Agriculture Organization (FAO), there has been a rise in the cultivation of mushroom at the rate of 1.05–1.24% every year from the year 2009 to 2018, which is accompanied by an increase in its consumption (Popa-Vecerdea and Oancea 2020). *Agaricus bisporus* is the most commonly consumed mushroom species. Other popular mushroom species are *Pleurotus* spp. and *Lentinus edodes*, with China being the largest producer of these mushrooms, followed by countries such as the United States, the Netherlands, Poland, Spain, and France (González et al. 2020).

21.3.2.2 Mycoproteins

Another important source of protein derived from microorganisms is mycoproteins. These are foods produced by the continuous fermentation of the filamentous fungus *Fusarium venenatum* and are rich in protein and fiber with low fat content. Mycoproteins were discovered after several years of study and search for new sources of protein for human consumption and are commercially produced in western countries as an important component of the diet (Denny et al. 2008).

Mycoproteins are rich in essential amino acids, vitamins, carotenes, and carbohydrates. The dietary fiber content of these mycoproteins consists of β-glucans and chitin-poly-*N*-acetyl glucosamine, which are appropriate in a normal healthy diet. Moreover, the low-fat composition consisting of favorable fatty acids such as linoleic acids, linolenic acids, and polyunsaturated fatty acids along with

high zinc and selenium percentage makes the mycoproteins a nutritionally rich food. However, compared to meat, the levels of vitamin B12 and iron are lower in mycoproteins (Hashempour-Baltork et al. 2020).

The fermentation technology can be modified to give these proteins a meat-like texture and taste and is the main ingredient in many protein-rich Quorn foods such as ready meals, minced meat, fillets, sausages, and chicken, as well as pastries, nuggets, burgers, and pies available in the USA and European markets (Denny et al. 2008). The fermentation process is followed by the downstream process of steaming, followed by chilling and freezing, resulting in a texture of mycoprotein similar to that of the chicken breast due to its fiber composition (Finnigan et al. 2019). This makes them a suitable alternative to animal proteins, i.e. meat, which have the limitation of time, energy, and cost. Since the mycoproteins are produced from agricultural waste, they have the advantage of being environmentally friendly (Hashempour-Baltork et al. 2020).

Mycoproteins were first approved for commercial use as a food ingredient by the UK Ministry of Agriculture, Fisheries and Food in 1983 and have been present in the market since 1985. Savor Pie under the brand name Quorn was the first commercial product with mycoprotein as an ingredient. The US Food and Drug Administration (FDA) also gave the designation "Generally Recognized as Safe" and introduced mycoproteins in their food products. In the present scenario, Quorn production reaches 25,000 dry masses per year with sales of 21 million (Matassa et al. 2016) and is consumed in 17 countries with approximately 5 billion servings to date (Finnigan et al. 2019).

21.3.2.3 Probiotics

The use of fermented dairy products to promote health was known long before knowledge of the existence of probiotics was discovered. However, with the development of the concept of probiotics, there has been intense research into probiotics and their use as functional foods. These are live microorganisms with health benefits when consumed in desired amounts and are not intended to have adverse effects on humans. They can be made with a pure single or mixed culture of microorganisms that interact with the normal microflora of the gastrointestinal tract and improve its properties for better digestion of food. Commonly used probiotic species include *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, and *Streptococcus* (Ozyurt and Ötles 2014).

Probiotics are not only a good source of various micronutrients, vitamins, minerals, etc., but also have numerous health benefits as they form active compounds such as phytonutrients, lipids, prebiotics, etc., which makes them an important functional ingredient in foods. In addition, they help balance and maintain a healthy gut microbiota, alleviate intestinal discomfort, and increase immunity (Jankovic et al. 2010). They also have the properties of lowering blood ammonia levels and cholesterol levels with the synthesis of vitamin B, increasing calcium absorption, and lactose utilization (Ozyurt and Ötles 2014). They are used in pharmacy as therapy to treat various diseases such as diabetes, obesity, diarrhea, liver diseases, allergies, cancers, etc. (Putta et al. 2018).

Table 21.2. Different fermented food products with probiotic activity

Food products	Probiotic organism present	References
Dairy based		
Yogurt	*Bifidobacterium* and *Lactobacillus acidophilus*	(Chen et al. 2017a)
Kefir	*Lactobacillus kefiranofaciens*, *Kluyveromyces lactis*, *K. marxianus*, *Torula kefir*, *Saccharomyces cerevisiae*	(Farnworth and Fu 2006; Chen et al. 2017b)
Fruits and vegetable based		
Sauerkraut	*Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Pediococcus dextrinicus*	(Beganović et al. 2011)
Kimchi	*Leuconostoc carnosum*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus sakei*	(Kim et al. 2017)
Cereal and Soya based		
Miso	*Tetragenococcus halophilus*, *Aspergillus oryzae*	(Kumazawa et al. 2018)
Tempeh	*Rhizopus oligosporus*, *Rhizopus oryzae*	(Chang et al. 2009)
Natto	*B. coagulans*, *B. subtilis*	(Azimirad et al. 2017)
Meat based		
Fermented sausages	*Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium* spp.	(de Macedo et al. 2012)
Nham	*Lactobacillus plantarum*	(Chokesajjawatee et al. 2020)

Most probiotics are derived from either human or animal gastrointestinal tract and are used in the food industry to produce fermented dairy products. Dairy-based products such as desserts, mayonnaise, butter, ice cream, powdered milk for infants, etc., formulated with the culture of these probiotics are being introduced as wellness brands in the global market (Cruz et al. 2009). Moreover, with the increasing demand for functional foods in the market, other nondairy products are also being manufactured with probiotics as an important ingredient. Numerous plant-based products such as fruit- and vegetable-based beverages and juices, cereal-based puddings, yoghurts, as well as meat-based products are produced either using probiotics for their fermentation or supplemented as starter cultures for commercial application (Song et al. 2012; Neffe-Skocińska et al. 2018). The most commonly available probiotic foods are yoghurt, kefir, yakult, kombucha, and sauerkraut (Putta et al. 2018) (Table 21.2).

21.3.2.4 Algae and Cyanobacteria

The microalgae are organisms with unicellular or multicellular nature and are one of the important carbon fixers worldwide. The microalgae in the marine environment are an important part of the aquatic food chain as they are the major primary producers of the marine ecosystem and the main source of food for aquatic organisms. Therefore, they are rich in numerous nutrients and have been used as food and in therapy since time immemorial (Udayan et al. 2017). Cyanobacteria are a similar phototrophic group of microorganisms found in various habitats and are also

known as blue-green algae. They are also used as a nutrient-rich food source in several parts of the world (Abed et al. 2009).

These organisms are an abundant source of bioactive compounds, and their use as dietary supplements is said to have great health benefits ranging from antiviral, antibiotic, and anticancer properties to lowering cholesterol level and weight loss. They are also said to be antidiabetic and help improve immunity and are therefore referred to as superfoods (Henrikson 1989). Both algae and cyanobacteria are either harvested directly from their habitat or cultivated in a controlled manner for their commercial use as dietary supplements (Gantar and Svirčev 2008). Some of the algae used nutritionally are *Haematococcus pluvialis*, *Chlorella*, *Nannochloropsis*, etc. *Chlorella* is a great protein source with protein production efficiency 50 times more than other protein sources (Goswami et al. 2021; Mehariya et al. 2021). It contains essential amino acids, vitamins E, C, and K, biotin, PABA, folic acids, choline, etc, and is used as a dietary supplement in the form of powder and tablets. *Haematococcus pluvialis* is used as a color additive and is the largest source of astaxanthin, while *Nannochloropsis* contains poly-unsaturated fatty acids like omega-3 fatty acid and is even used for their commercial production (Saini et al. 2021; Udayan et al. 2017). The cyanobacterial strains of *Nostoc*, *Spirulina*, and *Anabaena* are consumed in various countries such as the Philippines, Chile, Peru, and Mexico. *Arthrospira platensis* is considered the largest source of vitamin B12 and is also very rich in protein, β-carotene, riboflavin, thiamine, etc., while *Nostoc commune* has a high nutritional value for the presence of dietary fiber. The *Spirulina* is also used for the production of beer in a cost-effective manner under the brand name Spirulina beer and the other one being named as anti-age beer (Kovač et al. 2013). Other than acting as a human food supplement, cyanobacteria have also shown their potential as feed for aquaculture. In India, the *Phormidium valderianum* with nontoxicity and high nutritional value is used as a complete feed source for aquaculture (Abed et al. 2009).

21.4 Conclusion and Future Prospects

Microbes have wide applications in agriculture and food system and are essential in developing strategies to achieve the goal of sustainability. More and more research studies are being conducted to discover new applications and the mechanisms behind them. Numerous microbial strains are already being used commercially, for example, as biofertilizers, biocontrol agents, biopreservatives, and food supplements. However, the understanding of the full potential of these organisms for the development of agriculture and the food industry and the proper implementation of this knowledge is yet to be realized. The development of biotechnology and modern techniques can be used for increasing the efficiency of these organisms, in addition to their large-scale production and the development of action plans for putting this knowledge into practice.

Acknowledgments The authors would like to thank the Department of Microbiology, Sikkim University, for providing the computational infrastructure and central library facilities for procuring references.

Conflicts of Interests Authors does not have any conflicts of interests to declare.

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Microbes in Resource and Nutrient Recovery via Wastewater Treatment

22

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Abstract

Resource and nutrient recovery from wastewater is an essential task to achieve multiple goals such as degradation of toxic organic pollutants prior to discharge in natural water bodies, generation of useful biomass and bioproducts, elimination of waste sludge, etc. Classically, microbes have been used in the biological treatment of industrial wastewater particularly in the aeration tank and anaerobic sludge digester. Of late, several technologies have emerged for simultaneous wastewater treatment and nutrient recovery. In this regard, photosynthetic microorganisms such as purple phototrophic bacteria, cyanobacteria, and a consortium of green microalgae and methanotrophs have garnered significant research interest to treat a variety of wastewater, recover nutrients, and upcycle them to generate value-added products (biofuels, pigments, and feed-grade single-cell protein). The application of bioelectrochemical systems (microbial fuel cells and microbial desalination cells) and fabricated biogenic nanoparticles (BNPs) for oxidizing and adsorbing a wide range of pollutants help in wastewater treatment. In this chapter, we intend to discuss the role of various microorganisms in simultaneous wastewater treatment and synthesis of valuable bioproducts, the diversity and significance of electroactive microorganisms in bioelectrochemical cells for diverse wastewater treatment, resource recovery, and energy generation.

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_22

Finally, the advantages and challenges in BNP-based wastewater treatment would also be discussed.

Keywords

Wastewater · Nutrient recovery · Bioelectrochemical systems · Microbial fuel cells (MFCs) · microbial desalination cells (MDCs)

Abbreviations

BNP	biogenic nanoparticles
MDC	microbial desalination cells
MFC	microbial fuel cells
SCP	single-cell protein
SSF	solid-state fermentation.
NSAIDs	nonsteroidal anti-inflammatory drugs
PAHs	polyaromatic hydrocarbons
TP	total phosphorous
AE	aeration
AG	agitation

22.1 Introduction

An enormous rise in population, massive industrialization, rapid urbanization, and excessive utilization of resources are some of the environmental concerns which have overburdened natural resources including water and soil (Avtar et al. 2019; Kumar et al. 2018). In order to meet the needs of a growing population, the unregulated utilization of resources and unabated discharge of the pollutants and wastewaters into water bodies have deteriorated the quality of water making it unfit for drinking and agricultural purposes (Khalid et al. 2018; Kumar et al. 2019). Microorganisms are present in the environment as a very crucial factor for maintaining a balance among different facets of the ecosystem. In a broader sense, the term biodegradation refers to the cleansing act of microorganisms or decomposers to remove waste materials from the environment (Tahri et al. 2013). The term "mineralization" is used when microorganisms convert a substrate or a pollutant to a relatively safer product. The industrial revolution undoubtedly brought a drastic change in the lifestyle of humankind; however, since the eighteenth century, an enormous amount of pollutants and recalcitrant synthetic materials have been accumulating in the ecosystem particularly wastewater as a result of industrialization (Kumar et al. 2019). Many industries such as textile, paper, leather, food, etc., discharge waste materials in the water bodies which contain synthetic materials such as dyes (Saratale et al. 2011). In other sectors such as agriculture,

animal husbandry, biomedical, etc., for the enhancement of production, different synthetic materials such as pesticides, herbicides, and antimicrobial agents are being used for a pretty long period that led to conditions such as the accumulation of compounds known as xenobiotics. Xenobiotics are unwanted chemicals that accumulate in the body of an organism and if unmetabolized may reach the toxic level in the body (Hodgson 2012). Many microorganisms reportedly produce exoenzymes that have the capacity to degrade polymeric substances and other pollutants (Ahmed et al. 2018; Agrawal and Verma 2020a; Agrawal et al. 2018; Verma and Madamwar 2002a; Verma and Madamwar 2002b; 2002c). In Table 22.1, some selected enzymes involved in the biodegradation of pollutants are listed.

22.2 Use of Microorganisms in Wastewater Treatment for Removal of Toxic Substrates

The exploitation of microorganisms for the degradation of different potential hazardous materials in wastewater is a thrust area of research in environmental biotechnology. The contribution of microorganisms in the aforementioned field is elaborated below.

22.2.1 Microorganism-Mediated Dye Removal in Wastewater

In many industries like textile, plastics, paper, food, etc., dye is a frequently used material for color enhancement. Annually approximately 0.7 million tons of dye is manufactured, out of which around 10–15% is disposed of which increases the biological and chemical oxygen demand of water bodies (Dahiya and Nigam 2020). Based on their chemical structure and the chromophore present, the chemists reported 20–30 dye groups. Out of these groups, azo dyes, anthraquinone dyes, triarylmethane, and phthalocyanine dyes are the most important ones (El Sikaily et al. 2012). Many dyes contain xenobiotic functional groups such as aromatic sulfonic acid which is water-soluble that makes the dyes very difficult to remove (Knapp and Bromley-Challoner 2003). There are many adverse effects of dyes in the ecosystem as well as on the health of different organisms. Dyes are proven to be carcinogenic and have negative effects on photosynthesis and germination (Saratale et al. 2011; Ghodake et al. 2009; Kumar et al. 2021). Most of the higher organisms were found to be susceptible to the adverse effects of dyes. On the contrary, it was found that some fungi and bacteria could grow in substrates that contain a very high concentration of dye. Agriculture wastes are very good adsorbers of dyes (Dahiya and Nigam 2020; Agrawal and Verma 2020b; Chaturvedi et al. 2013a). The technique of solid-state fermentation (SSF) is utilized to ferment dye-adsorbed agriculture wastes, and it was found that the color intensity imparted by the dye particles significantly decreased due to fermentation by the fungi *Coriolus versicolor* and *Phanerochaete chrysosporium* (Robinson and Nigam 2008) Ref. Many bacterial strains are also found to be responsible for dye removal. It was found that

Table 22.1 Selected enzymes involved in the biodegradation of pollutants and their producers

Major biodegrading enzyme(s)	Producing microorganisms	Substrate(s)	Reference
Laccase	*Agaricus bisporus*, *Cerrena unicolor*, *Pleurotus eryngii*, *Trametes hirsuta*	Aromatic and aliphatic amines, hydroxyindoles, carbohydrates, and inorganic/organic metal compounds	Baldrian (2006); Giardina et al. (2010); Yang et al. (2017)
Monooxygenase and glucuronidase	*Alcaligenes faecalis*, *Staphylococcus aureus*, *Proteus mirabilis*	Nonsteroidal anti-inflammatory drugs (NSAIDs)	Murshid and Dhakshinamoorthy (2019)
Azoreductase, laccase, and peroxidase	*Pseudomonas* sp., *Bacillus subtilis*, *Geobacillus* sp., *Escherichia coli*, *Rhabdobacter* sp., *Enterococcus* sp.	Azo dye	Sarkar et al. (2017)
Phenol hydroxylase, catechol 2,3-dioxygenase	*Halomonas* sp., *Kocuria* sp.	Phenol	Li et al. (2019a, 2019b)
Lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, and versatile peroxidase	White-rot fungi, viz., *Funalia trogii*, *Irpex lacteus*, *Pleurotus pulmonarius*	Dye stuffs	Asgher et al. (2008)
Alkene monooxygenase, epoxyalkane coenzyme M transferase	*Dehalobacter restrictus*, *Geobacter lovleyi*	Chloroethenes	Mattes et al. (2010)
Acrylamidase	*Cupriavidus oxalaticus*	Acrylamide	Bedade et al. (2019)
Manganese peroxidase, lipase, laccase	*Cunninghamella elegans*, *Bjerkandera* sp., *Aspergillus niger*	Polyaromatic hydrocarbons (PAHs)	Kadri et al. (2017)
Alkane hydroxylase, alcohol dehydrogenase	*Geobacillus thermoparaffinivorans* and *Bacillus licheniformis*	Long-chain n-alkanes	Elumalai et al. (2017)
Carboxylase, pyrethroid hydrolase, esterase	*B. cereus*, *A. niger*, *Klebsiella* sp.	Pyrethroid insecticides	Cycoń and Piotrowska-Seget (2016)
Laccase, laccase-like multicopper oxidase (LMCO)	*Aspergillus flavus*, *Galleria mellonella*	Polyethylene microplastic	Zhang et al. (2019a, 2019b)

Enterobacter sp. was responsible for the degradation of crystal violet (Roy et al. 2018). There are bacterial strains that also degrade azo dyes like acid orange which are frequently used in textile industries (Singh et al. 2014). Even some yeast strains

are also involved in the decolorization of azo dyes (Jafari et al. 2013). Anthraquinone is another type of dye that was reported to be adsorbed by different strains of bacteria. It was observed that the assimilation of anthraquinone dyes is a function of the composition of the bacterial cell wall. Many bacteria having functional groups such as carbonyl, carboxyl, and amino on their cell surface are capable of better adsorption (Du et al. 2012). From the abovementioned and the other previously published reports, we can assume that the process of microorganism-mediated dye removal involves two processes: adsorption of the dye on the cell wall of the microorganisms and degradation of the chromophoric group present in them, thereby reducing the color intensity of the dyes (Agrawal and Verma 2019; Verma and Madamwar 2003).

22.2.2 Removal of Chemical Pollutants

Microorganisms which are abundantly present in the ecosystem are responsible for the degradation of organic pollutants released from various sources. Microorganisms may be obligate aerobes, obligate anaerobe, or facultative anaerobe, and depending upon the optimum concentration of oxygen and water, they degrade different metabolites. The presence of pollutants in the soil or water arises stress conditions for the microorganisms, and under stress conditions, they sometimes alter their metabolic pathways and start degrading chemical pollutants (Suteu et al. 2013). Controlled utilization of biodegradation led to the removal of chemicals such as halogenated and nonhalogenated organic compounds, hydrocarbons, nitrogen compounds, metals, radionuclide, etc. (Amedea et al. 2007). One of the most important pollutants that contain different hydrocarbons is petroleum and its products which create a great threat to the flora and fauna of the surrounding area. There are many reports of microbial-mediated decontamination of petroleum-polluted soil. Different bacterial strains were found to be involved in the decontamination process such as *Alcaligenes* sp., *Bacillus* sp., *Pseudomonas*, *Staphylococcus*, *Rhodococcus* sp., etc. (Mao et al. 2012; Tang et al. 2013; Lee and Cho 2009). The mechanisms by which the microorganisms degrade petroleum hydrocarbons are also studied in detail. It was observed that the main mode of decontamination is either by emulsification of oil or by assimilation into the metabolic pathways of bacteria (Suteu et al. 2013). Many enzymes are involved in the process of utilization. For example, in the case of the bacteria *Pseudomonas putida*, the enzyme toluene dioxygenase helps in the conversion of trichlorotoluene into simpler compounds like glyoxylate and formate (Wackett and Hershberger 2001).

Apart from petroleum hydrocarbon-related pollution, heavy metal pollution is another arising concern. Different heavy metal cations such as selenium, chromium, uranium, arsenic, and mercury are released into the environment by different industries. Although heavy metals are normally present in the earth's crust, anthropogenic activities increase the concentration of most of the heavy metals, and as a result, different organisms get exposed to them very easily. However, these metal cations cannot be directly removed. But different microorganisms produce different

enzymes and secretions which bound heavy metals, thus excluding them from the food chain (Suteu et al. 2013). Bacteria such as *Enterobacter*, *Azospira*, *Rhodobacter*, etc., are found to be involved in the conversion of selenium oxyanions into elemental selenium (Han and Gu 2010). Both hexavalent and trivalent chromium have a significant environmental impact. Bacteria such as *Arthrobacter*, *Pseudomonas*, and *Corynebacterium* were found to be involved in the reduction of chromium under appropriate conditions (Dey et al. 2014). Bacterial strains belonging to *Corynebacterium* were reported which were found to be resistant to arsenic stress, and they metabolize using different classes of the enzyme arsenate reductase (Lim et al. 2014).

Radionuclides are unstable elements released from the nuclear industries. It was reported that bacteria *Geobacter metallireducens* and *Shewanella oneidensis* can reduce uranium(VI) (Francis 1994). These bacteria could convert more toxic radioisotopes into less toxic and soluble ones such as uranium(IV) or technetium (IV) (Roh et al. 2015).

22.2.3 Bioremediation of Agricultural Livestock Industry-Generated Pollutants

In order to improve crop yield, different organic and inorganic compounds are being used for decades which has led to the accumulation of those products in the environment. The compounds include fertilizers, pesticides, herbicides, antibiotics, etc. Nitrogen- and phosphorus-based fertilizers lead to a condition known as nutrient pollution that results in the eutrophication of water bodies such as rivers, lakes, and ponds. Electroactive bacteria have the capacity to transfer electrons to a soluble electron acceptor without the help of any intermediate electron transfer shuttle (Li et al. 2020). A microbial electrochemical system (MES) is involved in this process, and using this system ammonium and phosphate can be recovered simultaneously.

Pesticides are used in agricultural fields for the removal of pests and weeds. Glyphosate is one of the most frequently used herbicides for the removal of annual and perennial weeds. It has been used for over five decades in fields, and its accumulation could pose a resinous threat to the environment (Wang et al. 2016). Works have been going on glyphosate degradation by bacteria, and it is reported that different strains of *Achromobacter*, *Comamonas*, *Ochrobactrum*, and *Pseudomonas* can degrade glyphosate (Firdous et al. 2017). The biochemical pathway responsible for this degradation was found to be sarcosine pathway (Zhan et al. 2018). Coming to other pesticides, they are often found to be resistant to degradation and tend to be retained in the environment and the living system. There are reports which indicate that different strains of *Bacillus*, *Exiguobacterium*, *Pseudomonas*, *Serratia*, and *Acidomonas* could successfully degrade pesticides such as aldrin, lindane, DDT, endosulfan, pentachlorophenol, etc. (Okeke et al. 2002; Lopez et al. 2005; Bidlan and Manonmani 2002).

The discovery of antibiotics has brought the revolution to antimicrobial therapy on only in medicine but also in other sectors such as agriculture, animal husbandry, etc. However, the never-ending use of antibiotics for more than four decades led to the accumulation of antibiotic residues and resulted in antibiotic resistance (Tacconelli et al. 2018). Both broad- and narrow-spectrum antibiotics are recovered from an environmental sample such as a river or seawater, soil, etc., which are carried by sewage water. Nontarget microorganisms such as gut microbiota are easily targeted by these types of discharged antibiotics and can lead to the total change in the microbiome of exposed animals. The microbial bioremediation of antibiotics can rely on the adsorption of antibiotics on extrapolymeric substances produced by bacteria (Zhang et al. 2018). It can also be attained by administering antibiotic-resistant genes such as beta-lactamase in the treatment group (Ng et al. 2019).

22.2.4 Role of Microbes in Nutrient Recovery from Wastewater

The untreated wastewaters generated in agriculture, sewage, and industries contain organic and inorganic compounds and heavy metals (HMs) besides being a rich source of nutrients like phosphorous (P) and nitrogen (N) in the form of orthophosphates, polyphosphates, nitrites, nitrates, and ammonia (Sood et al. 2015). Various biological, chemical, and physical approaches have been used for wastewater treatment and nutrient recovery (Crini and Lichtfouse 2019). The physical and chemical methods are usually costly, require high input of energy, and add different unwanted toxic substances to the water bodies (Udaiyappan et al. 2017). In this context, the engagement of plants and microorganisms for wastewater treatment, referred to as phytoremediation, has been regarded as a sustainable alternative for wastewater treatment and nutrient recycling. Microorganisms like cyanobacteria, microalgae, fungi, and bacteria are advantageous over large plants in terms of higher wastewater remediating and nutrient recycling efficiency and lesser biomass productivity (Mohsenpour et al. 2020; Romanis et al. 2020).

22.2.5 Role of Cyanobacteria in Nutrient Cycling via Wastewater Treatment

Cyanobacteria are a diverse group of photoautotrophic prokaryotic microorganisms with complex metabolism, lesser nutritional requirements, and better adaptability to fluctuating external environmental parameters. Due to the photoautotrophic mode of nutrition and their ability to utilize nitrogen and phosphorous from wastewaters, they have been regarded as an inexpensive alternative for wastewater treatment and nutrient recycling (Arias et al. 2020a; Chaturvedi et al. 2013b). Cyanobacteria have been used for a wide range of purposes like wastewater treatment, nutrient recycling, removal of HMs, and significant reduction of biological oxygen demand (BOD) and chemical oxygen demand (COD) (Honda et al. 2012). It has been

reported that cyanobacteria can assimilate different forms of P and N (NH^{4+}, NO^{2-}, NO^{3-}, and PO_4^{3-}) for growth, thus playing a significant role in municipal wastewater treatment and maintaining the structure and function of the ecosystem via nutrient cycling (Wang et al. 2012). Cyanobacterial culture of *Spirulina* (*Arthrospira*) has been utilized for removal of NO^{3-}, PO_4^{3-}, and NH^{4+} from secondary effluents of pig wastewater under tropical conditions. The total protein content from the harvested biomass of *Spirulina* was found to be 48.9% ash-free dry weight, while N and P removal was found in the range 84–96% and 72–87%, respectively (Olguín et al. 2003). The simultaneous treatment of municipal wastewaters and production of by-products in the form of carbohydrates using nitrogen-fixing soil cyanobacterial cultures were tested by Arias et al. (2020b). The study reported removal of more than 95% total nitrogen, more than 93% total organic carbon, more than 82% total inorganic carbon, and total phosphorus in the range of 35–78% from municipal wastewaters using an inoculum mostly dominated by *Nostoc* sp., *Tolypothrix* sp., and *Calothrix* sp. Singh and Thakur (2015) evaluated cyanobacterial endolith *Leptolyngbya* sp. ISTCY101, for simultaneous wastewater treatment and biodiesel production in semicontinuous mode. It was reported that the endolith produced 25% (% w/dw) of lipids which are mainly comprised of saturated and unsaturated fatty acids. It was also demonstrated that N and P were removed from the wastewaters at the rate of 4.37 mg L^{-1} d^{-1} and 1.01 mg L^{-1} d^{-1}, respectively. Nowadays, cyanobacterial-bacterial aggregates are being utilized for wastewater treatment due to their high removal rates of both organic and inorganic pollutants as well as nutrient recycling. The photosynthetic microorganisms (cyanobacteria) release oxygen for bacteria which degrade organic pollutants within wastewaters (Munoz and Guieysse 2006). The synergistic action of cyanobacterial-bacterial aggregates was reported by Papadopoulos et al. (2020) for the bioremediation of brewery wastewater using a cyanobacterial-bacterial consortium dominated by cyanobacterium *Leptolyngbya* sp. and *Chroococcus*-like. The study demonstrated that the cyanobacterial-bacterial consortium removed about 80%, 70%, and 90% of nitrates, orthophosphate and total phosphorus, and ammonium, respectively from the wastewater.

22.2.6 Role of Microalgae in Nutrient Cycling via Wastewater Treatment

Microalgae are photosynthetic microbes that enhance the oxygen content of the wastewater bodies, thereby increasing the rate of wastewater treatment and nutrient recycling either solo or in consortia of other microorganisms (Jia and Yuan 2016; Mehariya et al. 2021; Goswami et al. 2021). Microalgae-based wastewater treatment is advantageous over conventional methods in terms of its wide range of applications, viz., removal of nutrients including carbon (C), N, P, etc., HMs remediation, reduction of BOD and COD, removal of coliform bacteria, and detoxification of xenobiotic substances (Li et al. 2019a; Li et al. 2019b). Furthermore, microalgae show rapid growth, require comparatively less space for growth as well

as disposal, act as a rich source of bioactive by-products, and the residue can be used as biofertilizer and animal feed as well (Cai et al. 2013). It has been reported that by utilizing a microalgal-based approach for wastewater treatment, the energy consumption is reduced to half as compared to the conventional approaches. Additionally, 90% recovery of nutrients from wastewater through the microalgal-based system has been reported also (AciénFernández et al. 2018). Microalgae species show better adaptability to different wastewater systems and, thus, have been used for nutrient recycling via the treatment of livestock, industrial, agro-industrial, and municipal wastewaters (Abdel-Raouf et al. 2012). Yu et al. (2019) demonstrated potential nutrient removal from anaerobic digestion (AD) effluent by employing two microalgal species, *Chlorella vulgaris* (CV) and *Chlorella protothecoides* (CP). They reported that both CV and CP completely removed NH^{4+}-N (initial concentration 40 mg/L) without residual NO^{3-}-N or NO^{2-}-N from AD within 10 days using batch mode along with the lipid production as a by-product. Microalgae *Chlorella protothecoides* were used for N and phosphorous (P) recovery and biodiesel production by treating brewery wastewater. A significant amount of about 90% and 96% of total phosphorus and nitrogen were removed from the brewery wastewater, respectively. Additionally, *C. protothecoides* accumulated 1.88 g L^{-1} of biomass, and the total fatty acid content of 35.94 $\pm$ 1.54% of its dry cell weight was also reported (Darpito et al. 2015). Kothari et al. (2013) demonstrated the removal of 90% nitrate, 74% nitrite, 70% phosphate, and 90% ammonia from dairy industry wastewater by algal strain *Chlamydomonas polypyrenoideum*. Ajayan et al. (2015) studied the phytoremediation of tannery wastewater (TWW) using microalgae *Scenedesmus* sp. The algal biomass reduced the nutrients NO_3 and PO_4 by 44.3% and 95%, respectively. The study demonstrated the removal of nitrogen and phosphorus from municipal wastewater using microalgae species *Chlorella vulgaris*. The strain removed about 94–96% COD, 92–100% phosphorus, and 98–100% nitrogen, along with 2.2 g L^{-1} of biomass production (Mujtaba et al. 2018). The nutrient removal capacity of green microalgae C. *vulgaris*, *Scenedesmus obliquus*, *Parachlorella kessleri* TY, *Chlorococcum* sp. GD, and *S. quadricauda* from undiluted cattle farm wastewater was demonstrated by Lv et al. (2018). Among the studied microalgal strains, *C. vulgaris* exhibited maximum nutrient removal capacity. It was reported that the nutrient removal efficiency of *C. vulgaris* from cattle farm wastewater was found to be 91.24–92.17%, 90.98–94.41%, and 83.16–94.27% for COD, total phosphorous (TP), and NH^{4+}-N, respectively. A 90% removal of COD and 100% removal of nutrients from undiluted dairy wastewater by microalgae *Acutodesmus dimorphus* were reported by Chokshi et al. (2016). Benítez et al. (2019) reported the employment of native microalgae Ecuadorian *Chlorella* sp., for nutrient cycling from synthetic secondary wastewater. The project was conducted in two sets (1) with aeration (AE) and (2) with agitation (AG). It was observed that nitrogen and phosphorous removal efficiencies were 52.6% for AG and 55.6% for AE and 67.0% for AG and 20.4%for AE, respectively.

22.2.7 Role of Methanotrophs in Nutrient Cycling via Wastewater Treatment

Methanotrophs are a group of Gram-negative bacteria that oxidize methane in the environment and uses it as a sole source of carbon and energy (Cai et al. 2016; Mayr et al. 2020). Methane is regarded as the second most potent greenhouse gas after carbon dioxide. In addition to other anthropogenic sources, wastewater and wastewater treatment plants are regarded as important reservoirs of greenhouse gases including methane. Methanotrophs, in this regard, play a vital role in mitigating the emission of methane from wastewaters, along with the release of value-added products in the form of bio-methanol, nitrogen removal, and biopolymers synthesis (Al Sayed et al. 2018). Kim et al. (2020) studied nitrogen recovery from ammonium-rich wastewater (reject water) by the simultaneous action of methanotrophs and methanol-dependent (*Methylophilus*) bacterial consortia. In this study, methanotrophs and *Methylophilus* removed ammonium by 59% through autotrophic assimilation, while the methanol generated by methanotrophs was assimilated by *Methylophilus,* simultaneously. A co-culture of microalgae-methanotroph provides a sustainable approach for nutrient recovery and fuel production from wastewater. The study carried out by Roberts et al. (2020) demonstrated that the co-culture of *Chlorella sorokiniana-Methylococcus capsulatus* removed about 71% nitrogen and 164% phosphorous from the wastewater along with the production of biomass. Zha et al. (2021) reported the nutrient removal and single-cell protein (SCP) production from sewage sludge employing a consortium of methanotrophic bacteria predominated by *Methylomonas* sp. and *Methylophilus* sp. They observed a dry weight yield (biomass) of about 0.66 ± 0.01 g-DW/g and 11.54 ± 0.12 g-DW/g of CH4 and $NH4^{+}$, respectively, with a protein content higher than 41%w/w. Some of the microbes found useful for wastewater treatment and nutrient recovery have been summarized and listed in Table 22.2.

22.3 Microbial Fuel Cells and Microbial Desalination Cells in Wastewater Treatment

22.3.1 Microbial Fuel Cells

Microbial fuel cells (MFCs) are bioelectrochemical systems that generate electricity by using electrons derived from oxidation or reduction reactions catalyzed by bacteria. Such bacteria are referred to as "electrogenic" owing to their ability to exchange electrons from microbial outer membrane to the conductive surface (Ramanjaneyulu and Reddy 2019; Agrawal et al. 2019). There are two types of electrogenic bacteria: (1) electrode-reducing bacteria and (2) electrode-oxidizing bacteria. While the former transfers electrons to an anode by oxidizing organic compounds or substrates into carbon dioxide, the latter reduces substances in the cathode chamber by using electrons from the cathode (Clark and Pazdernik 2015). In aerobic chamber, electrode-oxidizing bacteria can reduce oxygen to water. In

Table 22.2 Role of cyanobacteria, microalgae, and methanotrophs in wastewater treatment and nutrient recovery

S. No.	Type of wastewater	Organism	Nutrient recovery	By-product	Reference
1	Dairy wastewater	*Chlorella vulgaris*	Nitrogen, carbon	NM	D'Imporzano et al. (2018)
2	Cassava processing wastewater	*Arthrospira platensis*	Ammonia (100%), nitrite (100%), nitrate (91%), phosphates (55%)	NM	Araujo et al. (2020)
3	Synthetic wastewaters	*Chlorella vulgaris*	Nitrogen, carbon, phosphorous	Lipid	Ge and Champagne (2016)
4	Toilet wastewater	*Chlorella sorokiniana*	Nitrogen, phosphorous	NM	Fernandes et al. (2017)
5	Livestock and industrial wastewater	*Chlorella vulgaris*, *Spirulina platensis*	Nitrogen, phosphorous	Bioplastic	Zeller et al. (2013)
6	Municipal wastewater	*Leptolyngbya* sp.	Nitrogen, phosphorous	Biodiesel	Singh and Thakur (2015)
7	Aerated piggery wastewater (APW)	*Desmodesmus* sp. CHX1	NH_4-N (78.46%) Total phosphorous (91.66%)	Algal biomass	Luo et al. (2019)
8	Municipal wastewater	*Chlorella vulgaris*	N (9.8) and P (3.0) (mgl^{-1} $days^{-1}$)	NM	Cabanelas et al. (2013)

anaerobic conditions, sulfate $(SO_4)^{2-}$ or nitrate NO^{3-} can be reduced to sulfide and nitrite ions, respectively. Similarly, carbon dioxide can be reduced to methane or acetate. Two of the most studied electrogenic bacteria for MFCs are *Geobacter sulfurreducens* and *Shewanella oneidensis*. However, *Geobacter* is an obligate anaerobic bacterium and is extremely intolerant to oxygen. Similarly, *Shewanella*, although it can survive in oxygen, does not completely oxidize organic compounds. Therefore, it is also important to identify novel electrogenic bacteria that are capable of utilizing a broader spectrum of organic compounds for use in MFCs (Sacco et al. 2017). Interestingly, wastewater contains the organic substances that these electrogenic bacteria require to carry out the redox reactions, thereby providing an opportunity to generate clean energy and simultaneously remove pollutants. Although the application of MFCs for domestic wastewater treatment has been demonstrated under laboratory scale, the full-scale industrial application is hindered due to the high cost of the materials (anode, cathode, and the proton exchange membrane), the requirement of external energy input for starting the MFC circuit, and low buffering capacity of the wastewater. Nonetheless, tremendous advances have been made in wastewater treatment and energy generation research using MFCs (Guo et al. 2020).

22.3.2 Microbial Desalination Cells

Microbial desalination cell (MDC) is an emerging wastewater treatment technology that combines MFC and electrodialysis for desalination of water, generation of clean energy, and resource recovery (Saeed et al. 2015). It is based on the principle that when organic matter present in the wastewater is oxidized by electroactive bacteria into treated effluents, electricity is generated, and the ionic species migration (protons) within the system facilitates desalination (Kokabian and Gude 2019). MDC finds several applications such as desalination of sea and brackish water, generation of clean fuel (hydrogen), reducing the hardness of water by removing dissolved calcium and magnesium, remediation of groundwater, etc. (Sophia et al. 2016). Furthermore, using a properly designed MDC, the current-driven separation of volatile fatty acids, ammonia, and phosphorous from wastewater is possible. Thus, nutrient or resource recovery using MDC is an emerging area of interest, and current research is directed toward exploring its technical and economic feasibility (Liu et al. 2020). Several exo-electrogenic microorganisms that carry out anaerobic respiration such as denitrifying bacteria (*Pseudomonas*, *Ochrobactrum*), sulfate-reducing bacteria (*Desulfuromonas*, *Desulfobulbus*), and dissimilatory metal-reducing bacteria (*Geobacter*, *Shewanella*, *Geothrix*) have been reported to be associated with microbial fuel cells and could potentially be used as the exoelectrogen of choice in MDC system. Similarly, anoxygenic phototrophic bacteria (purple non-sulfur bacteria such as *Rhodoferax ferrireducens*) and photosynthetic *Rhodopseudomonas palustris* DX-1 have also been reported as electrogenic bacteria (George et al. 2020; Xing et al. 2008) that could be suitably employed as a biocatalyst in MDC. Several studies have reported simultaneous removal of organic pollutants and power generation using MFCs. A list of recent studies that demonstrated wastewater treatment using MFCs has been shown in Table 22.3.

22.4 Role of Fabricated Biogenic Nanoparticles (BNPs) for the Biosorption and Biodegradation of Toxic and Emerging Pollutants

Wastewater contains different types of pollutants and is of different origin and properties. The toxic pollutants are produced due to their use in industries, households, hospitals, and activities associated with farming (Kokkinos et al. 2020). The toxic and burgeoning pollutants of the water body are continuously crippling the traditional methods of wastewater treatments**.** The evolving water body pollutants mainly include heavy metals, organic and inorganic pollutants, pharmaceuticals and many pathogenic microbes, etc. The modern technologies to treat wastewater are not cost-effective. Hence the fabricated biogenic nanoparticles (BNPs) could be cost-effective alternative to treat wastewater because of their distinctive properties such as high catalytic reactivity and surface area for biodegradation and biosorption of evolving toxic pollutants of wastewater and recovery of essential nutrients present in wastewater. Different compounds from plants like

Table 22.3 Summary of MFC- and MDC-based wastewater treatment approach

Biocatalyst or microorganism	Wastewater source	Fuel cell type (MFC or MDC)	COD removal	Electricity generation	Reference
Halophiles (*Ochrobactrum*, *Marinobacter*, *Martelella*, *Rhodococcus*, and *Bacillus*)	Seafood	Air cathode MFC reactor	78 ± 1.5% TSS removal at 1.25 g COD/L organic load	980 mV, 570 mW/m^2, 600 mA/m^2	Jamal et al. (2020)
Bacillus and *Lysinibacillus* (when unconnected) *Pseudomonas aeruginosa* and other *Bacilli* classes (for stacked MFC) (anodic chamber)	Domestic wastewater	Continuous flow 40 Nos. stacked air cathode MFC	39% first module (first 10 MFC units) 74% during the last modules (13–40 MFC units)	4.9 V, 2500 mW/m^2, 500 mA/m^2	Estrada-Arriaga et al. (2018)
Shewanella oneidensis MR-1	Bamboo fermentation effluent	Batch dual-chamber MFC	75–83%	0.76 V, 0.3–0.6 W/m^2, and 1.7–2.7 A/m^2	Dai et al. (2020)
Geobacter and sulfate-reducing bacteria	Active sludge mixed with stimulated wastewater	Pyrite-based constructed wetland MFC	71.9%	24.77 mA/m^2 and 2.67 mW/m^2	Ge et al. (2020)
Chlorobia and deltaproteobacteria	Domestic wastewater	Algae biofilm MFC	80.2%	62.93 mW/m^2	Yang et al. (2018)
Sulfate-reducing bacteria (13.94% abundance) in anode	Mustard tuber wastewater (high salinity)	Biocathode MDC	Salt (97.4%), sulfate (99.7%), and nitrogen (99.8%) removal	0.51 mA and 0.64 mA	Zhang et al. (2019a, 2019b)
Proteobacteria and actinobacteria	Domestic wastewater	Three-chambered MDC	52%, 66% (salt removal)	8.01 W/m^3	Luo et al. (2012)
Anaerobic microbes (from WTP)	Petroleum refinery wastewater	Three-chambered MDC	64.0% (with PBS as catholyte)	827 mV (with seawater)	Sevda et al. (2017)

(continued)

Table 22.3 (continued)

Biocatalyst or microorganism	Wastewater source	Fuel cell type (MFC or MDC)	COD removal	Electricity generation	Reference
			70.5% (with acidified water)	738 mV (with 20 g/L NaCl)	
Mixed consortium (obtained from aerobic sludge of WTP)	Synthetic wastewater	Photosynthetic MDC using microalgae (*Chlorella vulgaris*) biocathode	64% (with 35 g/L TDS)	675 mW/m^3	Kokabian et al. (2018)
Oscillatoria sp.	Dairy wastewater	Photosynthetic biocathode MDC	80.2 ± 0.5 (COD removal) 65.8 ± 0.5% (desalination efficiency)	652 ± 10 mV, 44.1 ± 1.0 mW/m^2	Bejjanki et al. (2021)

Maximum electricity (mV), power density (mW/m^2 or mW/m^3), current density (mA/m^2)

steroids, terpenoids, alkaloids, flavonoids, and saponin can be used for the fabrication of biogenic nanoparticles. These metabolites have to have reducing and stabilizing properties so they could be employed as the source of fabrication along with biogenic nanoparticles to enhance biosorption or biodegradation property and nutrient recovery from wastewater and could be an alternative to metal nanoparticles (Choudhary et al. 2018). Wastewater is a supply house of nutrients that can be recovered and used in agriculture and the production of energy. But the conventional methods of nutrient recovery have limitations like low recovery rate and use of toxic substances. Biosorption is the preferable method of recovery of nutrients from wastewater because of the high recovery rate (Bishoge et al. 2018). The fabricated biogenic nanoparticles increase the functional groups on the surface of sorbent for selective recovery of nutrients from wastewater (Beyene and Ambaye 2019). It has already been reported that biogenic nanoparticles fabricated with different bacterial strains are used to remove toxic pollutants of wastewater such as heavy metals, toxic dye, pigments, pesticides, and pathogenic microbes and also used for recovery of essential metal ions (Bilal et al. 2020). The main benefit of using bacterial strains fabricated biogenic nanoparticles is that bacterial strains can change the oxidation state of the metals and could be used for the recovery of nutrients from polluted water bodies. The main mechanisms involved for recovery and removal or biodegradation of pollutants from wastewater include oxidation in combination with ion exchange, electrostatic attraction, and reduction due to metabolic activities, etc. The fabricated biogenic nanoparticles removed metal ions by the adsorption process. The adsorption of the metal ion depends on the reducing agents with opposite charges produced by bacterial strains and specific functional groups such as hydroxyl, carboxyl, methyl, amide, etc. These functional groups are involved in electrostatic interactions and ion exchange and thereby remove or recover metal ions from wastewater by precipitation. Moreover, the fabricated biogenic nanoparticles can change the oxidation state of toxic dye and inorganic and organic pollutants present in wastewater. In addition to the removal and recovery of metal ions by adsorption mechanism, the fabricated biogenic nanoparticles have exceptional biodegradation properties for different types of organic pollutants and dye present in the wastewater. Also, the emerging pollutants like steroid hormone, pharmaceuticals, and pesticides were easily biodegraded by using fabricated biogenic nanoparticles. So biologically fabricated nanoparticles can be used as an efficient tool for wastewater treatment (Ngoepe et al. 2020).

The biogenic synthesis of nanoparticles has application in the field of sensors to detect or monitor toxic pollutants like heavy metals, pesticides like malathion and chlorpyrifos, or nutrients present in wastewater and soils (Das et al. 2018; Siddiqi et al. 2016).

There are many applications of fabricated biogenic nanoparticles in the field of a wastewater treatment system which includes removal of organic and inorganic contaminants, pharmaceutical pollutants, nitro compounds, dyes, and arsenic from wastewater. There is a report of arsenic removal and its adsorption on the surface of fabricated iron nanoparticles. Ammonia, phosphate, and various other pollutants of wastewater can be easily degraded or removed by using biogenic nanoparticles.

Antibiotics are the main pharmaceutical pollutants of wastewater that pose a serious threat to human health and can be removed by fabricated biogenic nanoparticles (BNPs). The processes like biologically promoted and regulated mineralization are used for the synthesis of biogenic nanoparticles where nanoparticle growth is controlled by organisms. The magnetosomes are produced by magnetotactic bacteria, which are used for the recovery of metals and removal of metals from wastewater produced by industrial activities.

The efficiency percentage of metals and nutrients recovery from wastewater is always lower than expected due to the presence of many unwanted pollutants that act as a barrier (Nasrollahzadeh et al. 2020). Hence, biogenic nanoparticles are coated with soluble biological materials for absorbing nutrients to increase the recovery rate. Biogenic gold nanoparticles act as photo-nanocatalysts to recover essential metal ions present in wastewater and remove harmful dyes like methylene blue and o-nitroaniline (Patel et al. 2020).

The unique characteristics like less toxicity and eco-friendliness of the fabricated phytogenic magnetic nanoparticles are used to remove pollutants from wastewater (Yaqoob et al. 2020). The absorption and accumulative properties of microbes and plants are exploited for the removal and recovery of both organic and inorganic pollutants from wastewater (Santhosh et al. 2016). The functional groups present in plant metabolites like amino acids, polyphenols, and polysaccharides can be used as capping and reducing agents during fabrication and help in nutrient recovery and pollutant removal from wastewater (Ali et al. 2017).

The performance of fabricated biogenic nanoparticles (BNPs) is high due to higher surface area and catalytic activities. So they can be a suitable alternative for wastewater treatment both technically and economically.

22.5 Conclusion

Recent advances in microbe-assisted wastewater treatment are now seen as an opportunity to recycle waste efficiently and simultaneously avoiding environmental pollution. Microbe-assisted wastewater treatment works on an approach of degradation of toxic pollutants, minimizing waste sludge, biomass production for generation of energy and value-added product, etc. Microbes-based biological treatment of industrial wastewater is gaining interest, and the advent of several potential microbes and technology has improved the simultaneous wastewater treatment and nutrient recovery. In recent times several purple phototrophic bacteria, methanotrophs, cyanobacteria, and a consortium of green microalgae are being shifted to large-scale application for treatment of a variety of wastewater, to recover nutrients which further can be used as the substrate for the generation of value-added products (biomethane, bioethanol, carotenoids astaxanthin pigments, and feed grade single-cell proteins). Another approach to obtain clean water, energy, and nutrients from wastewater is by using bioelectrochemical systems such as microbial fuel cells (MFCs) and microbial desalination cells (MDCs). Such technologies rely on the metabolic activity of electrogenic bacteria that can oxidize a wide variety of

compounds using solid-state electrodes. Additionally, microorganisms are also used to fabricate biogenic nanoparticles (BNPs) for the biosorption and biodegradation of toxic and emerging pollutants from wastewater including heavy metal-contaminated water. BNPs have unique properties such as high catalytic activity and specific surface area that offer promising applications in wastewater treatment and resource recovery.

Conflict of Interest Statement The author declares no conflict of interest.

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Potential of CRISPR/Cas9-Based Genome Editing in the Fields of Industrial Biotechnology: Strategies, Challenges, and Applications

23

Ashwani Kumar Verma, Dixita Chettri, and Anil Kumar Verma

Abstract

The development of the system clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) represented a major breakthrough in industrial biotechnology. CRISPR/Cas9 is a highly orthogonal and versatile system and has stimulated research in the fields of metabolic engineering, systems biology, and synthetic biology. The CRISPR/Cas9 toolbox includes CRISPR editing, CRISPR activation, CRISPR interference, and CRISPR-mediated protein imaging. These basic tools can be used to model the genome and program the desired gene expression, paving the way for efficient genomic and transcriptomic manipulations to optimize the efficiency of microorganisms. These CRISPR/Cas9 techniques can be further improved for the overproduction of the desired enzymes. The method has been successfully applied to a number of industrially important microorganisms including bacteria, yeast, and filamentous fungi. However, the efficiency of the CRISPR/Cas9 system's gene processing is still unsatisfactory, and several versatile models of this method can be designed for better efficiency.

Keywords

CRISPR/Cas9 system · Industrial microorganisms · Genomic sculpting · Programmed gene expression · CRISPR editing · CRISPR-mediated protein imaging

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_23

23.1 Introduction

Genome engineering, the ability to precisely modify and manipulate desired DNA sequences, is at its peak. This potential of genome engineering methods to perform DNA sequence editing precisely and effectively has attracted research devoted to a wide range of biotechnological fields, including medicine, energy, and environmental studies. Programming and targeting of nucleases with site-specific DNA binding regions are such intriguing approach that enables scientists to perform experimental studies with improved performance, accelerated assembly of nucleases, low cost of genome manipulation, etc. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and meganucleases (homing endonuclease engineered) are methods developed by adapting DNA endonucleases that help to generate double-strand breaks (DSBs) at the target DNA sequence. ZFNs are generated by combining DNA cleavage domain with zinc finger DNA binding domain. Zinc finger of 30 amino acids forms a compact structure by folding around the zinc ion. This compact structure recognizes three base pairs in the DNA sequence. The structure must be optimized before assembly so that it can recognize the target DNA sequences. However, the accessibility of all sites is limited and requires a newly optimized protein for each target site. Therefore, the application of ZFNs is limited because polymorphisms and active sites cannot be targeted by enzymes (Roidos 2014). TALENs proved to be more advantageous over ZFNs as they can detect higher activity on their chromosomal target sequences (Gaj et al. 2013). Golden Gate cloning is the main high-throughput method developed for TALENs. This molecular cloning method uses restriction enzymes of type II, which bind to DNA at one site and cleave the adjacent site (Engler and Marillonnet 2014). However, similar to ZFNs, TALENs are also limited by the restricted accessibility of chromatin sites and off-target effects. The meganucleases approach involves a genetically engineered homing endonuclease that recognizes the target site. A recent addition to this list of true targeting tools is a method used with bacterial nuclease acknowledged as clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system. The method was described as an ideal tool for introducing double-stranded breaks into site-specific DNA sequences (O'Geen et al. 2015). In CRISPR/Cas9-mediated gene manipulation, the nuclease enzymes are guided by a small RNA molecule, approximately 20 nucleotides long, called short guide RNA (sgRNA or gRNA), to target the specific desired DNA site. This breakthrough genome manipulating tool offers several non-negligible advantages such as (1) targeting efficiency, (2) speed of assembly, (3) multi-targeting potential, (4) lower cost, etc.

CRISPR system is classified into two broad classes: class 1 and class 2. This classification is based on the selection of Cas protein and the effector modules architecture. The classes are further divided into 6 different types and 33 subtypes (Makarova et al. 2015, 2020). Class 1 system is divided into three types: types I, III, and IV. All three types mediate resistance to the invading elements with the help of a multi-membered effector protein complex consisting of 4–7 Cas proteins. The three types are distinguished by their unique and specific signature proteins: type I consists

Cas3 protein with nuclease and helicase domains, type III contains Cas10 with histidine aspartate (HD) catalytic domain and two palm domains, while type IV contains protein Cas8. Type III is again divided into four different types: types III A, B, C, and D. Unlike the class 1 systems, the class 2 system requires only a single protein to perform its natural function of CRISPR-mediated defense machinery. The class 2 system is again divided into three different types: types II, V, and VI. Similar to class 1, the classification is based on signature proteins selection: (1) type II consists Cas9 protein, (2) type V consists Cas12a, Cas12b, Cas12c, and Cas13a, and (3) type VI contains Cas13b and Cas13c. Among all, type II Cas9 protein is widely used in biotechnological applications and was first discovered from *Streptococcus thermophilus* (StCas9). Cas9 protein has been identified from a number of other bacterial members such as *S. pyogenes* (SpCas9), *Staphylococcus aureus* (SaCas9), *Francisella novicida* (FnCas9), etc. X-ray diffraction study of Cas9 protein of *S. pyogenes* revealed that the crystal structure contains two different structural lobes: a small nuclease lobe (NUC) and another lobe referred to as the large recognition lobe (REC) (https://www.rcsb.org/structure/4CMP). The REC lobe helps to recognize DNA sequences, while the NUC cleaves the recognized sequence. REC is divided into REC1 and REC2, while the large recognition lobe contains two catalytic domains: RuvC (cuts nontarget strand) and HNH (cuts the complementary strand) (Nishimasu et al. 2014; Parkhi et al. 2018).

Various applications of CRISPR/Cas9, such as silencing of competing pathways, integration of signaling pathways, development of tolerance to metabolic stress, and control of fermentation leading to overall strain improvement, have attracted numerous research efforts dedicated to enhancing the viability of industrial microorganisms in various biotechnological applications. However, no genetic engineering method is without limitations, and the CRISPR/Cas9 system also has certain drawbacks. The limitations mainly relate to the low efficiency of genome editing and possible off-target effects. The random mutations called off-target effects can affect other genes in the genome of the organisms, resulting in undesirable products. The effectiveness of this approach can be improved by addressing the drawbacks through the design of versatile and modified forms of CRISPR/Cas9 models (Fig. 23.1).

23.2 CRISPR/Cas9-Based Mechanisms

CRISPR/Cas9 technology was derived from the bacterial and archaeal adaptive immune system, and it contributes toward the endogenous adaptive immunity in bacterial genomes by approximately 40% and in sequenced archaeal members by about 70% (Burstein et al. 2016). This system provides adaptive immunity by following a conserved series of processes that includes adaptation, expression, and interference. These three different stages provide DNA encoded RNA-mediated targeting of specific sequences in the exogenous nucleic acid. After the infection of the host, the adaptation phase begins with the invasion of the host cell by the foreign DNA (such as phage DNA). The invading DNA is cleaved into short DNA

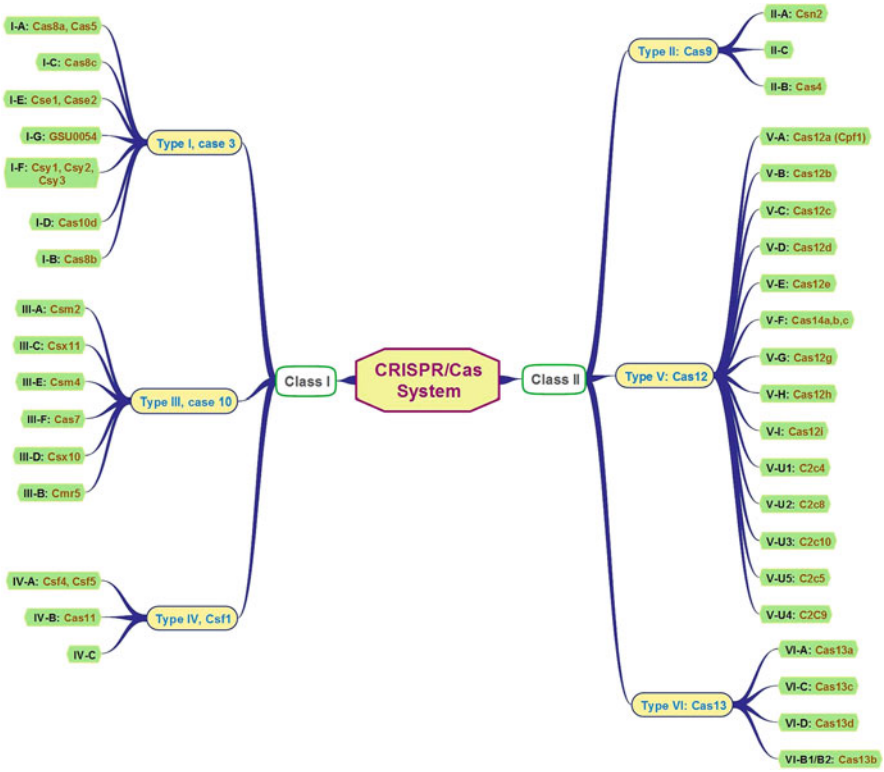

Fig. 23.1 Classification of CRISPR/Cas system based on presence of unique signature proteins (Makarova et al. 2015, 2020; Toro et al. 2019; Yan et al. 2019)

fragments called protospacer by the various *cas* genes. The protospacer fragment is inserted into the tandem array of CRISPR locus in naturally occurring systems. During the expression phase, the CRISPR locus with integrated novel "spacer" can be transcribed to produce a precursor CRISPR RNA (pre-crRNA). Type II CRISPR system contains pre-crRNA that remains complex with CRISPR-associated protein (Cas9) and transactivating CRISPR RNA (tracrRNA). RNA hybrid thus formed between pre-crRNA and tracrRNA is recognized by RNase III enzyme, and this helps to produce the processed and mature crRNA-tracrRNA dual-RNA hybrid. The mature crRNA combines with Cas9 and the dual-RNA: Cas9 complex is directed to the DNA site. The single effector protein, i.e., Cas9 protein, functions as RNA-guided double-strand DNA endonuclease and creates double-stranded breaks (DSBs) at the target DNA site. Unlike CRISPR type II system, there is a requirement of a large multifactor complex of Cas9 protein in types I and III to carry out the function of crRNA-guided DNA targeting. The interference phase includes the after events when the foreign DNA invades again during reinfection. The dual-RNA/Cas9

complex recognizes the sequence that is complementary to the spacer and cleaves the invading DNA, thus inhibiting host cell infection.

The favorable attributes of CRISPR/Cas9 technique have been extended to gene editing and gene regulation in the number of industrial microorganisms including bacteria and fungi. For genomic manipulations, the crRNA and the tracrRNA of the dual-RNA/Cas9 complex can be reinstated by the application of a single RNA molecule designated single guide RNA (sgRNA or gRNA). The complex can recognize the sgRNA only in the presence of a specific protospacer adjacent motif (PAM). It is required that the PAM sequence must be present immediately downstream of the gRNA target sequence. For transcriptional regulations, a deactivated form of the single effector protein Cas9 (dCas9) is used that lacks the endonuclease activity. The dCas9 can be used in several transcriptional regulation tools.

23.2.1 CRISPR/Cas9-Mediated Genomic Manipulation

Versatility of CRISPR/Cas9 technique for gene deletion as well as gene introduction was initially suggested by Jiang et al. in 2013. They provided the initial proof of the concept using two bacterial cells, *Streptococcus pneumoniae* and *Escherichia coli*. Their method involved reprogramming the hybrid dual-RNA/Cas9 complex specificity by altering crRNA sequence and thus made single as well as multinucleotide changes on the editing templates. Multiplex mutagenesis was enabled following the simultaneous use of the two crRNAs. When the method was combined with the recombination technique, the desired mutation was observed in about 100% of *S. pneumonia* cells and about 65% of *E. coli* cells (Jiang et al. 2013a; b).

The formation of DSBs at the desired DNA site makes CRISPR/Cas9 system more preferable over the conventional gene editing tools. The introduction of DSBs improves recombination rates when applied along with a suitable DNA donor molecule. Two DNA repair mechanisms are applicable to repair the DSBs created by Cas9 protein: nonhomologous end-joining (NHEJ) repair and homologous repair (HR). NHEJ repair mechanism is capable of introducing deletions or insertions (indels) at the desired DNA site, while HR mechanisms use a foreign DNA donor molecule to allow the recombination of the exogenous DNA at the target site. The efficiency of homologous directed repair (HDR) is usually lower due to the competing DSB repair pathways such as the NHEJ repair. The DNA repair mechanisms help to introduce the desired sequence in the target site. The sgRNA required for CRISPR/Cas9-mediated genome alterations can be constructed by programming a nearly 20 base pair complementary region with NGG PAM matching loci directly into the heterologous CRISPR array and combined with crRNA and tracrRNA to produce the sgRNA. The sgRNA when combined with Cas9 protein can be used to target the desired site on DNA and create DSBs. The repair of the DSBs following the NHEJ repair mechanisms can result in gene knockout (KO) by introducing frameshift mutation in the open reading frames (ORFs). In CRISPR/Cas9 system, this KO capability leading to the elimination of undesirable genes can be programmed to redirect the metabolic pathway. This approach can contribute toward

several competing reactions involved in undesirable functions such as breakdown of precursor metabolites or undesired breakdown of carbon source. KO of multiple genes can be conducted following an effective method of single-step transformation of multiple sgRNAs. This method is very beneficial for studies that require multiple knockout events such as the study of the epistatic relationships in metabolic flux, di- or polyploid yeasts, or potentially inessential functions of the members of gene family, etc. The method was applied by Jakočiūnas et al. (2015) for the multiplex metabolic engineering of yeast *Saccharomyces cerevisiae* using CRISPR/Cas9 genome editing tool. They employed the Uracil-Specific Excision Reagent (USER) cloning technique (Jensen et al. 2014) for the accommodation of multiple sgRNA expression cassettes in a 2 μm vector backbone. The USER cloning technique allowed smooth and simultaneous integration of several fragments of DNA into the USER site at the backbone of the vector used. They investigated the capacity of formation of DSBs at the target site using multiple sgRNA expression by comparing the efficiency of transformation for a set of single, double, triple, quadruple, as well as quintuple sgRNA plasmids to a non-sgRNA cassette containing the plasmid. From the results obtained, they demonstrated that there is no observed decrease in the transformation efficiency when expressing multiple sgRNA on the plasmid backbone compared to non-sgRNA containing plasmid transformation efficiency.

Many microorganisms lack the NHEJ repair mechanism, and hence the CRISPR/Cas9-mediated DSBs might be lethal. In such cases, DSBs repair is highly reliant on the other repair mechanisms including HR, and also the possibility of alternative end-joining repair mechanisms has been suggested in some bacteria lacking the NHEJ repair pathway. Thus, the success of CRISPR/Cas9 system, in this case, is dependent upon the sufficient tuning of Cas9 function and the homologous repair mechanism. An approach in the case of NHEJ-deficient microorganisms involves λ-RED-mediated recombination to increase the lower efficiency of HR mechanism. CRISPR/Cas9 technique is utilized along with λ-RED recombinase and a short DNA donor that has the capability to span the DSB formation to carry the gene knockout, or small local changes can be introduced without leading to DSB-mediated cell death (Donohoue et al. 2018).

23.2.2 CRISPR/Cas9-Mediated Transcriptional Regulations

An important step toward the efficient synthesis of a specific desired compound is the fine-tuning of the different biosynthetic pathways. Traditional strategies used are dependent on the small number of characterized promoters which might be strong, weak, or can be inducible. The permanent genetic alterations such as gene KO may not always ensure efficient regulations of metabolic flux and thus may not give a satisfactory yield of the desired product. The method of constitutive expression of endogenous and exogenous genes stands with a number of limitations. Such expression of genes may result in a decreased cellular growth, harmful cytotoxic effects, and might hinder the phenomenon of feedback inhibition. CRISPR/Cas9-mediated

transcriptional control is an effective method that allows efficient metabolic pathway flux control in terms of both gene transcription timing and satisfactory levels. This method escapes the negative effects of overexpression of the desired gene. CRISPR/Cas9 tools for transcriptional regulation exhibits its unique function of transcriptional regulation and involve a deactivated form of single effector protein, Cas9, which is designated as catalytically dead Cas9 or dCas9. Mutation in critical residues in the two nuclease domains catalytically inactivates Cas9 protein and thus converting it into dCas9 (Fig. 23.2).

One such study included H840A and D10A mutations in two domains, HNH and RuvC, in the dCas9 protein. The result of such mutations showed the loss of DNA cleavage activity (endonuclease activity), but the protein retained the binding activity to DNA (Qi et al. 2013). dCas9 combines to the sgRNA and helps to develop two CRISPR transcriptional regulation tools: CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa), as described below:

23.2.2.1 CRISPR Interference (CRISPRi)

CRISPRi tool for transcriptional control has shown high potential for modulating the level of several gene expression. CRISPRi enables this modulation by using the inactivated dCas9 protein. The gRNA/dCas9 complex inhibits the binding of RNA polymerase enzyme once it is bound or is present in the immediate vicinity of transcriptional start site (TSS). Thus co-expression of dCas9 with sgRNA can interfere with transcriptional events such as RNA polymerase binding, transcription elongation, and/or may inhibit binding of transcriptional factors. This tool for transcriptional regulation can repress multiple genes simultaneously and reversibly. Temporal control and switching on/off of repressor system in CRISPRi are enabled with the help of an inducible promoter that controls dCas9 and sgRNA (Perez-Pinera et al. 2013). The reversibility and the induction of CRISPRi were tested by encoding both dCas9 and a monomeric red fluorescent protein (mRFP)-specific sgRNA (NT1) which were kept under influence of an inducible anhydrotetracycline (aTc) promoter. The measurement of time period of inducer-regulated mRFP was also conducted. During the cell growth, the high stability of mRFP protein allowed the diluted protein to limit the decrease of the fluorescent signal rate. The result data obtained indicated a quick response of the system for the inducer present (Qi et al. 2013). The CRISPRi sgRNA-mediated silencing of the gene is also very specific and does not exhibit any off-target effects. The specificity was demonstrated by a whole-transcriptome shotgun sequencing study for cells transformed with dCas9 with or without the sgRNA co-expression. The result obtained in presence of sgRNA targeted to mRFP (NT1) showed mRFP transcript as the only gene showing a reduction in the abundance. Within sequencing errors, no other gene was observed showing a significant change in the expression followed by the addition of sgRNA (Qi et al. 2013). The degree of gene repression using CRISPRi showed only modest results in studies conducted in human and yeast cells. A significant increase in gene repression is observed by the combination of dCas9 and effector domains with regulatory functions. The human codon-optimized dCas9 encoding gene was created from *Streptococcus pyogenes*. The resulting dCas9 was fused with two copies of

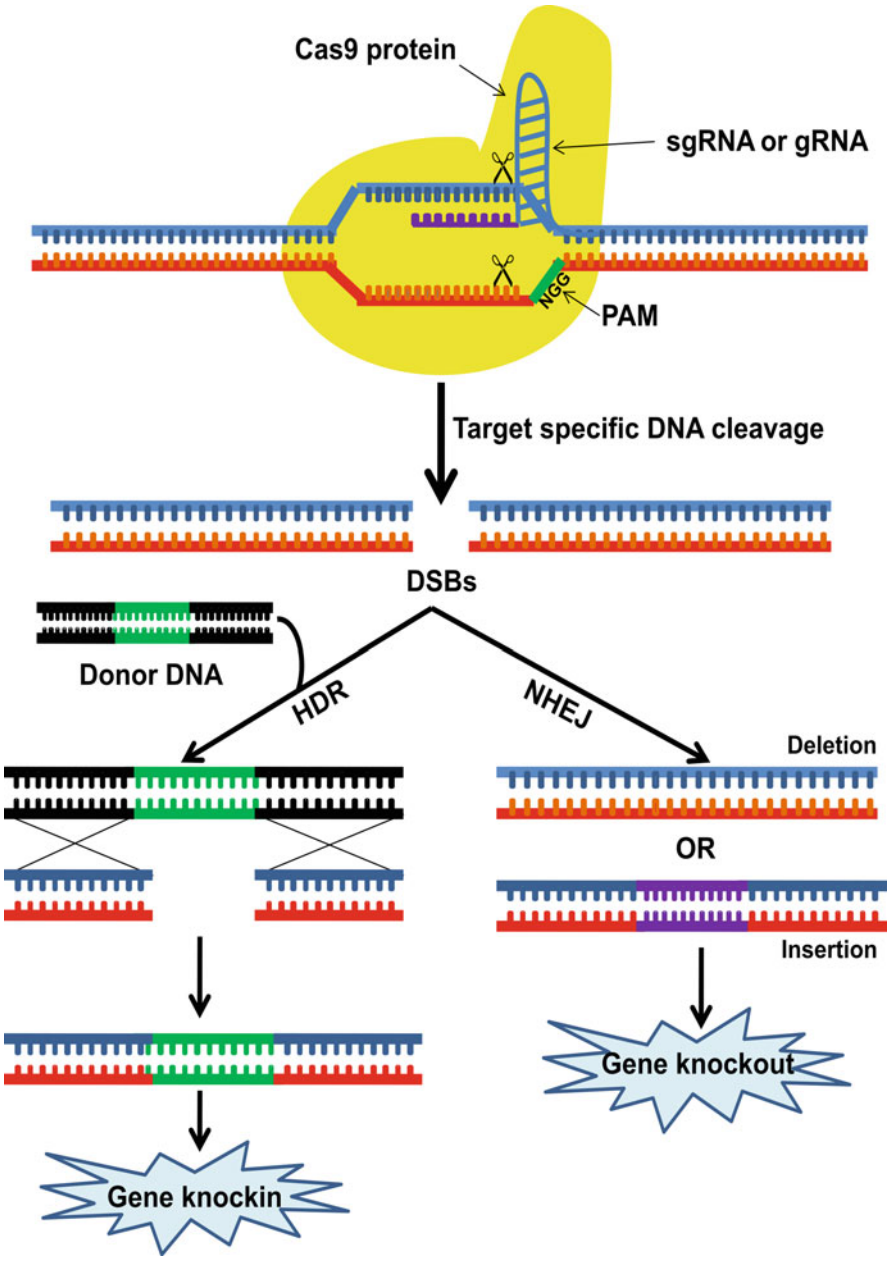

Fig. 23.2 Mechanism of CRISPR/Cas9-mediated genomic manipulation

nuclear localization sequence (NLS), a single copy of blue fluorescent protein (BFP), and HA tag. The modified dCas9 was further fused with the repressive domains involved in chromatin modifications including the domain of Kox1, chromo shadow (CS), and Kruppel-associated box (KRAB). The results for fusion with KRAB showed a decrease in GFP signal by fivefold, while dCas9 without any fusion was able to show repression of about twofold (Gilbert et al. 2013).

23.2.2.2 CRISPR Activation (CRISPRa)

CRISPR activation (CRISPRa)This tool of CRISPR/Cas9 transcriptional regulation helps in the activation or upregulation of the target genes. This method requires the fusion of catalytically dead dCas9 with the transcriptional activation domain. This system helps to boost the rate of transcription of selected genes even under the control of the weak promoter. dCas9 transcriptional activator complex thus formed enhances the target gene expression by targeting the upstream region of the desired gene and recruiting the RNA polymerase to activate the desired gene transcription. Such transcriptional activators usually include the key domains, for example, transactivator domain in herpes simplex virus protein 16, i.e., VP16, several copies of VP16 including VP64 or VP160, or p65 transactivator domain of nuclear factor kappa B. The fusion of the dCas9 protein with RNA polymerase enzyme subunits in *E. coli* showed effective recruitment of the other subunits of the same enzyme, thereby improving the target protein production by about 2.5-folds (Bikard et al. 2013).

dCas9 protein when combined with multiple copies of transactivator domains can result in a significant increase in transcriptional activation. Using such a method of multi-copy fusion of transactivator domains, a number of efficient CRISPRa systems have been created including SAM, VPR, SPH, SunTag system, etc. Multiple gene activation has been successfully approached by the construction of scaffold RNA (scRNA). Simultaneous activation and repression of the desired gene have been accomplished using CRISPR activation and interference tools by adding different RNA scaffolds on the 3′-end of sgRNA. This technique is called bidirectional regulation. RNA scaffolds can help in the bidirectional regulation of multiple genes in a biosynthetic pathway (Zalatan et al. 2015).

23.3 CRISPR/Cas9-Mediated Genetic Engineering in Industrial Microorganisms

Low-cost renewable resources, including plant biomass and organic waste, can be converted into valuable products by a range of industrial microorganisms. The potential of such microorganisms as industrial strains can be enhanced by various genetic engineering methods. CRISPR/Cas9 method proves to be highly efficient over conventional time and labor-intensive methods of genetic manipulation. This method can be successfully implemented in various microorganisms, including bacteria, filamentous fungi, and yeasts, for overall strain improvement, thus resulting

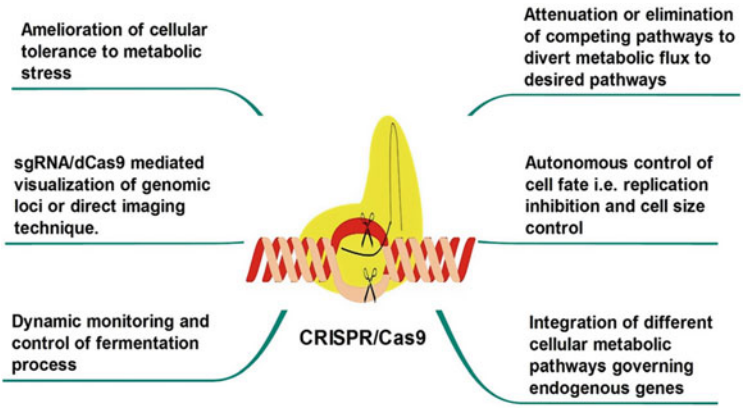

Fig. 23.3 CRISPR/Cas9-based genetic engineering for overall industrial strain improvement

in desirable microbial cell factories (MCFs). Applications of CRISPR/Cas9 system in various industrial microorganisms are discussed (Fig. 23.3).

23.3.1 Bacteria

CRISPR/Cas9 approaches have shown satisfactory results in industrially relevant bacterial strains. Such bacteria have a high potential for producing value-added products such as chemicals, drugs, and biofuels. *Escherichia coli* has been the center for many CRISPR/Cas9-based studies aimed at increasing the efficiency of this system in bacteria. The initial approach of using two plasmids for subsequent expression of Cas9 protein and gRNA proved to be lethal to the cell, so the idea of combining both components into a single plasmid containing the pBAD promoter was later proposed. Before genome editing could proceed in the transformed *E. coli* cells, both Cas9 and sgRNA were repressed by glucose to remove the burden on cellular metabolism. Cas9 and sgRNA were then further induced by a temperature-sensitive repA101ts replicon and arabinose substrate. This repression allowed the transformed cells to grow on glucose-containing plates, and the cells were readily edited under 2 g/L arabinose induction (Zhang et al. 2020). Simultaneous multiple loci genome editing was further improved by the development of CRISPR/Cas9-assisted multiple genome editing (CMGE) method. This approach involves the assemblage of all functional key components within replicable plasmids. An inducible expression system controls the Cas9 gene expression, decoupling the genome editing process from the transformation process. Therefore, only a single transformant is required after the transformation process, and this single cell would eventually divide to form a large population before the editing process is initiated by inducing the functional components. The CMGE technique was successfully implemented in *E. coli* by Feng et al. (2018) using three plasmids: (1) pRedCas9 plasmid, which contains the λ-RED recombination and Cas9 system

and is held under pBAD promoter that drives genome cleavage and homologous recombination; (2) pMgRNAs plasmid, which expresses multiple loci targeting gRNAs; and (3) donor DNA plasmid (pDonorDNAs) with donor DNA cassettes. The technique when performed for two and three loci genome editing showed high efficiency with 100% and 88.3% respectively, while 30% efficiency was shown for four loci genome editing (Feng et al. 2018). The recombination technique of the Cas9 system could be further exploited to construct a CRISPR-enabled trackable genome editing tool (CREATE). CREATE was used for the transformation of multiple libraries of plasmid-derived recombination templates simultaneously into *E. coli* cells. CREATE strategy helped in the introduction of numerous variations in ribosomal binding sites in *E. coli* for the development of isopropanol pathway genomic libraries. This approach helped in constructing and testing nearly 1,000 cells in a span of just a few days with the best achieving an isopropanol titer of 7.1 g/L within the time period of 24 h (Liang et al. 2017).

In addition to *E. coli*, the CRISPR/Cas9 method has been successfully implicated in other bacterial members including *Bacillus*, *Clostridium*, *Corynebacterium*, *Lactobacillus*, *Streptomyces*, etc. Recently, a hyperbutanol-producing strain, *C. saccharobutylacetonicum* N1–4, was engineered using genetic engineering methods based on CRISPR/Cas9 system. Genetic modification in *C. saccharobutylacetonicum* was performed based on the functionality of CRISPR/Cas9 system adapted for the bacterium *C. beijerinckia*. Low genetic efficiency with mutation rates of less than 20% was observed when two important genes for acetate (*pta*) and butanol (*but*) production were evaluated and selected for single and double mutations. Genetic efficiency was increased from 20% to about 75% using an optimized PJ23119 promotor for the gRNA expression. Deletion of the *pta* and *but* genes then showed the highest production of butanol of 19.0 g/L from batch fermentation (Wang et al. 2017). Besides constructing robust strains for solvent production, CRISPR/Cas9-mediated technique can also aid in the production of bulk chemicals from a variety of bacteria. CRISPR/Cas9 and recombination techniques were coupled to perform genetic engineering in *Corynebacterium glutamicum* L-glutamate overproducer ATCC 13032 to develop an efficient method to screen for optimal knockout gene combinations that lead to overproduction of γ-aminobutyric acid (GABA). GABA is a highly beneficial industrial chemical with broad applications in nylon production, animal feed, and pharmaceuticals. The strategy was used to iteratively perform the sequential knockout of one or more genes. Synthetic single-stranded oligodeoxyribonucleotides (ssODNs) were incorporated into genome using RecT recombinase, and the negative mutants were counter-selected by a Cas9-sgRNA-RNP complex, resulting in DSB formation. Seven mutant strains were generated by these methods, which were later used to demonstrate the combined deletion effects of three candidate genes, *Ncg11221* (encoding L-glutamate exporter), *gabT* (encoding GABA transaminase), and the *gabP* gene (encoding GABA permease), all are involved in GABA production. Such genome editing methods lead to accelerated metabolic engineering in the bacterium with a combinatorial 27.5 ± 0.3 g/L GABA yield (Cho et al. 2017). Many other works dedicated to bacterial gene editing applying CRISPR/Cas9 approach in

bacteria have established the method as a solid basis for future industrial developments (Fokum et al. 2019).

23.3.2 Yeasts and Filamentous Fungi

Filamentous fungi and yeasts are widely known for their extensive industrial applications. Yeasts play a pivotal role in the production of several industrial products such as biofuels, biocatalysts, chemicals, food additives, biopharmaceuticals, etc. The ability to withstand harsh processing conditions such as high temperature and low pH makes yeasts potential industrial strains. Several filamentous fungi are widely applied in the production of important metabolites including antibiotic medicines, polyunsaturated fatty acids (PUFA), pigments, and organic acids. Traditional genetic engineering methods give low efficiency results, especially in filamentous fungi owing to the complexity introduced by thick chitinous cell wall, cellular differentiation, lack of suitable plasmids, etc. CRISPR/Cas9 approach came as a breakthrough for genomic engineering studies in yeasts and filamentous fungi.

An important model for various CRISPR/Cas9-based studies is *Saccharomyces cerevisiae*. CRISPR/Cas9 system efficiency in this microorganism was verified using CAN1, an endogenous genomic negative selection marker that encodes for the enzyme arginine permease (DiCarlo et al. 2013). The efficiency of genome manipulation was further improved by Uracil-Specific Excision Reagent (USER) cloning technique. This is a ligase-free technique that helps to assemble a number of DNA fragments into a single construct. This technique includes USER cloning, its fusion, and the USER cassette-free (UCF) USER fusion. It has been used to perform the assembly of multiple gRNAs into a single plasmid, resulting in efficient gene disruption. The USER technology could be used for one-step promoter engineering of about 1–5 target loci. High single- or fivefold gene editing efficiency rates of approximately 50–100% could be achieved with this marker-free technology. However, co-transformation with plasmids containing sgRNA showed lower efficiency, which is one of their setbacks (Lian et al. 2018). The lower efficiency rates of co-transformation in USER technology could be improved by another approach, the homology-integrated CRISPR system (HI-CRISPR). Here, a HR template of about 100 base pairs was combined into the 5′-end of the crRNA sequence. This achieved high multiplex knockout efficiency (87%) of *CAN1*, *LYP1*, and *ADE2* (Bao et al. 2015).

The CRISPR/Cas9 approach has been applied for integrating expression cassettes containing heterologous genes at more efficient rates than traditional techniques. Three genes isolated from *Scheffersomyces stipitis*, *XYL1*, *XYL2*, and *XYL3*, the genes encoding xylose reductase, xylitol dehydrogenase, xylukinase enzymes, respectively, were integrated into *S. cerevisiae* for their efficient expression. The ability of CRISPR/Cas9 to generate DSBs and subsequent HR was exploited for deletion of unwanted genetic elements: *PHO13* and *ALD6*. Three desired genes present in a single linearized DNA segment were used to co-transform yeast with

sgRNA plasmids. This method was used to construct mutants within 3 weeks that showed overexpression of the desired genes (Tsai et al. 2015).

Filamentous fungi including *Aspergillus niger*, *Trichoderma reesei*, *Penicillium chrysogenum A. oryzae*, etc., have been applied for decades for commercial enzymes and metabolites production. *Trichoderma reesei* is a potential MCF due to its ability to degrade lignocellulosic biomass and its wide commercial application. Liu et al. (2015) first attempted to perform genetic modification on *T. reesei* following the CRISPR/Cas9 system. The establishment of this approach was demonstrated after site-specific optimization of codon and in vitro transcription process. *Cas9* gene isolated from *S. pyogenes* was optimized and subsequently linked to the SV40 nuclear localization signal. In vitro sgRNA synthesis successfully performed KO of *laeI* (endogenous putative methyltransferase gene) and *vibI* (encodes VIB1, which links glucose signaling pathway and carbon catabolite repression) genes. The result of this study suggests CRISPR/Cas9-based method as a promising method for gene manipulation in *T. reesei* and possibly in other filamentous fungi (Liu et al. 2015). CRISPR/Cas9-based genome manipulation technology was employed to perform the knockout of *SxlR* gene. This gene negatively regulates xylanase activity in *T. reesei*, and its deletion showed an increased expression of the gene encoding GH11 endoxylanase (Leynaud-Kieffer et al. 2019). Another important filamentous fungus is the cellulase enzyme producer *A. oryzae*. The low efficiency of genome editing in this fungus has been increased using CRISPR/Cas9 system. Multiple copies of AMA1 plasmid, which contains an autonomously replicating sequence, were introduced into the fungal strains, eventually leading to overexpression of Cas9 and sgRNA, increasing the editing efficiency in wild and industrial staining to approximately 50–100% (Katayama et al. 2019). Many such studies using CRISPR/Cas9 method performed on yeasts and filamentous fungi solidify this approach as a powerful tool to increase the efficiency of industrial strains (Table 23.1).

23.4 Limitations of CRISPR/Cas9-Mediated Applications

Despite the successful use of CRISPR/Cas9-mediated genome manipulation in a large number of industrial microorganisms, this approach to genetic engineering still faces some limitations. Major drawbacks of CRISPR/Cas9 tools include the absence of effective tools for the generation of sgRNA arrays and their delivery, higher risk for off-target mutations, low efficiency of gene editing, dependence on neighboring protospacer motifs, arrangement in plasmids or chromosomes, analysis of CRISPR-generated mutations, etc. Some important challenges of this breakthrough genetic engineering tool are discussed.

Table 23.1 Applications of CRISPR/Cas9-mediated genetic engineering in industrial microorganism: bacteria, filamentous fungi, and yeasts

Industrial microorganisms	gRNA	Cas9 protein	Gene editing efficiencies	References
Bacteria				
Escherichia coli	pMD19T plasmid (after sequencing recombinant product referred as repMD19T) and pTargetF plasmid	pCas plasmid with ParaBAD promoter	The gene recombination efficiency ranging between 0and 77.78%, when homology arms was 50–300 bp	Hou et al. (2020)
Clostridium cellulolyticum	gRNA containing P4 promoter ligated into pCR8/GW/ TOPO TA vector	A codon optimized *Cas9* fragments integrated into pLyc 017 vector	High gene editing efficiency of greater than 95%	Xu et al. (2015)
Clostridium autoethanogenum	SgRNA was introduced into $PMTL8_{3157}$ plasmid. Resulting gRNA with homology arms (HA) referred: psgRNA-adh-T_1_HA and psgrna-adh-T_2_HA	Codon optimized *Cas9* gene into pLZtet3no and $pIPL_{12}$ vectors. Resulting vectors: ptet3no-Cas9 and $Pipl_{12}$-Cas9	Efficiency of desired gene deletion improved to over 50%	Nagaraju et al. (2016)
Streptomyces albus	P_{gapdhp}-gRNA-T_{fd}	P_{rpsLp}-Cas9-T_{fd}	Targeted chromosomal deletion using rapid multiplex genome editing with efficiencies ranging between 70 and100%	Cobb et al. (2015)
Corynebacterium glutamicum	gRNA targeting *ldhA* gene inserted into pEC-$XK_{99}E$ plasmid under control of P_{lac} promoter	Plasmid pCas9 constructed by inserting P_{tac}/ P_{prpD2}-$Cas9^{I80bp}$-rfp cassettes into $pXMJ_{19}$	High gene deletion and gene insertion efficiencies with 60.0% and 62.5%, respectively, using plasmid-borne editing templates	Liu et al. (2017)

(continued)

Table 23.1 (continued)

Industrial microorganisms	gRNA	Cas9 protein	Gene editing efficiencies	References
Bacillus subtilis	gRNA targeting *ldhA* gene inserted into pEC-XK$_{99}$E plasmid under control of P_{lac} promoter	lacA5'-Cas9-tracRNA-lacA3'	Single and double gene mutations conducted with high efficiency of 100% and 85%	Westbrook et al. (2016)
Filamentous fungi				
Trichoderma reesei	gRNA cassettes with synthetic gRNA and ura5 gene target DNA ligated into the pMD-18 T and maintained in DH5α	*Agrobacterium tumefaciens* AGL1 plasmid under constitutive promoter Ppdc and inducible promoter Pcbh1 employed for expressing *Cas9 gene*	Single/multiple gene disrupting efficiencies of ≥93%/ 4.2–45%	Liu et al. (2015)
Aspergillus niger	cDNA006, *albA* gene targeting small guide RNA, (sgRNA001)	Codon optimized Cas9 inserted into pFC332 plasmid	Gene editing efficiencies reaching about 100%	Leynaud-Kieffer et al. (2019)
Candida albicans	5′ homology arm-SNR52 promoter-gRNA1-gRNA2–3'	Codon optimized Cas9. IDT gene blocks and genomic PCR employed to construct base *NEUT5L* locus integrating Cas9 drive-containing plasmids	Efficiencies ranging 25–100% for single and double gene disruption, respectively	Shapiro et al. (2018)
Sclerotinia sclerotiorum	pCRISPR/ Cas9-TrpC-Hyg vector used for sgRNA construction	Codon optimized Cas9, tef1 promoter	Gene disruption efficiencies ranging from 38% to 100%	Li et al. (2018)
Yeasts				
Schizosaccharomyces pombe	Plasmid pMZ252 constructed by assembling sgRNA, rrk1 promoter,	Plasmid pMZ222 constructed by assembling of two Cas9	85–90% single gene disruption efficiencies	Jacobs et al. (2014)

(continued)

Table 23.1 (continued)

Industrial microorganisms	gRNA	Cas9 protein	Gene editing efficiencies	References
	CspCL placeholder into pUR19N (OM473/ OM474)	fragments plasmid pART1		
Kluyveromyces marxianus	Hybrid RNA pol III promoters: SNR52, $tRNA^{Gly}$, and RPR1-$tRNA^{Gly}$ used for sgRNA expression	PTef1p-Cas9-*CYC1TT*	Highest single gene disruption efficiency of 66%	Löbs et al. (2017)
Ogataea polymorpha	pUC-HpSNR6a and pHP-gRNA	P_{TDH3}-Cas9	Single gene disruption efficiency of 45%	Numamoto et al. (2017)

23.4.1 Generation and Delivery of sgRNAs

An important challenge in CRISPR/Cas9-based genome modification is the generation and delivery of sgRNA. The use of RNA polymerase II to generate sgRNA is limited due to posttranscriptional modification at both ends of the mRNA molecules produced by this enzyme. In addition, mRNAs produced by RNA pol II are transported from the nucleus to cytoplasm, whereas CRISPR sgRNA requires access to the genome within the nucleus. Therefore, to circumvent this limitation, U3 and U6 promoters, transcribed by RNA polymerase III, were first used for this purpose. However, this approach to sgRNA production also has significant limitations. Both U3 snRNA and U6 snRNA are expressed ubiquitously as housekeeping genes. Therefore, they cannot be applied to generate the sgRNA required for the CRISPR/Cas9 system. Second, such promoters are difficult to find and characterize in a number of organisms, and furthermore, they are not suitable for routine experimental use due to the noncommercial availability of the enzyme RNA polymerase III. U3 and U6 promoters further limit the target sequence of CRISPR to G(N)20GG and A(N)20GG, respectively (Zhang et al. 2014). Gao et al. addressed this problem by designing an artificial PGR gene that transcribes RNA molecules that undergo self-catalyzed cleavage to produce sgRNA with ribozymes at both ends (Gao and Zhao 2014). In addition, more attention needs to be paid to the delivery of sgRNA to the intended genomic loci. Injection-based techniques, such as injection of Cas9/sgRNA-expressing plasmids (Gratz et al. 2013) and injection of RNA-CRISPR components (Bassett et al. 2013), are commonly used for delivery. However, the efficiency of delivery usually varies between different organisms, and more robust designs need to be developed for this purpose.

23.4.2 Dependence on Protospacer Adjacent Motif (PAM)

The specificity of the CRISPR/Cas9 approach applied to desired DNA sequence is based on complementarity between sgRNA and target sequence. A PAM sequence of 2–5 nucleotides is present immediately downstream of the target DNA sequence. Among several Cas9 orthologs, this sequence is found to vary. For example, in bacterium such as *S. pyogenes* and *S. thermophilus* type II, the PAM sequences present are NGG and NGGNG/NNAGAAW (Le Rhun et al. 2019; Agudelo et al. 2020). This PAM-dependent genetic engineering tool usually harbors limited frequencies of genomic target sites. For example, it is not difficult to search NAG PAM and NGG PAM for a target site every 8 nucleotides, but it can be quite limiting to search a specific target site every 32 and 256 nucleotides for NGGNG PAM and NNAGAAW PAM, respectively.

23.4.3 Low Efficiency and Off-Target Effects

In prokaryotes, the HR DNA repair mechanism is more suitable for genome editing processes than the NHEJ pathway. However, the HR pathway suffers from low editing efficiency, which needs to be addressed by developing certain versatile models that lead to improved CRISPR/Cas9 genome editing efficiency. The various versatile models that address this problem were discussed in Sect. 23.5. Another important problem for the broad applicability of CRISPR/Cas9 approach is the off-target mutations associated with it. Undesired mutations lead to off-target effects. Such unwanted mutations result from the presence of multiple highly homologous or identical sequences to the target DNA sequences in the genome of the microorganisms. These nontarget identical sequences are sometimes cleaved by the CRISPR/Cas9 method in addition to the target sequence, which can be lethal and lead to cell death. Therefore, research is focused on eliminating this problem of off-target mutations and associated cell toxicity. An important approach to solving this problem is to select a target sequence with a few off-target sites in the genome as possible. This strategy is pursued by identifying possible off-target sequences in the genome. For this purpose, a number of online tools were used, such as TagScan (Iseli et al. 2007) and Pattern Match (http://viewer.shigen.info/medakavw/patternmatch/) (both search up to two mismatch region) and BLASTIN and BLAT (both heuristic alignment online tools that are unable to search short nucleotides of about 20 nucleotides). Xiao et al. (2014) developed an open-source and downloadable online tool for the same purpose called CasOT. This tool was designed to search for off-target sequences or sites in any specific region of the genome based on PAM sequence and mismatch number in the seed as well as non-seed regions (Xiao et al. 2014).

23.5 Designing the Versatile Models of CRISPR/Cas9 System

Genome editing efficiencies of CRISPR/Cas9 approach can be improved by designing a number of versatile models. Some of such models have been described.

23.5.1 Enhancing the Efficiency of Homology-Directed Repair (HDR)

The main mechanism for CRISPR/Cas9-mediated gene editing is through the repair of DSBs using NHEJ or HDR pathways. NHEJ pathway is preferred over HDR by most industrial microorganisms, but the NHEJ pathway does not allow precise genome editing because it can introduce insertion/deletion mutations (indels). Such indels can lead to a shift in ORFs, causing the targeted gene to lose its function. Therefore, it is appropriate that the introduction of specific gene knock-ins or other targeted mutations be performed through the HDR pathway. Moreover, the low efficiency of the HDR pathway is a limitation for achieving effective gene editing. Therefore, studies are being conducted to favor the HDR pathway and increase its efficiency for effective and accurate gene editing. Genetic and chemical modulation methods have shown satisfactory results for favoring the HR pathway. One method involved the use of *ku70* deletion strains (Δ*ku70* strains) to mediate specific integration of marker-free donor cassettes using the HR pathway in a CRISPR/Cas9 study conducted in *Pichia pastoris* wild-type strain CBS 7435. Ku70 is an essential protein that, together with Ku80 protein, forms a Ku heterodimer that binds to DNA DSBs and is instrumental in the NHEJ repair pathway. The absence of the same would divert the repair of DSBs to the HR pathway. The CRISPR/Cas9 vectors used contained ribozyme enzyme-flanked sgRNAs designed to target Cas9 protein with different codon optimization and GUT1 sequence. Three different codon optimizations of Cas9, *PpCas9* (codon-optimized Cas9 from *P. pastoris*), *SpCas9* (codon-optimized protein Cas9 from *S. pyogenes*), and *HsCas9* (human codon-optimized Cas), were maintained under the control of a bidirectional promoter. Although an absolute number of transformed in Δ*ku70* strain is somewhat reduced, this technique achieved an integration efficiency of approximately 100%. In addition, placement of autonomously replicating sequence (ARS) in donor cassettes showed a 25-fold improvement in integration efficiency in the case of wild-type strains (Weninger et al. 2018). Another important approach to increase the efficiency of the HR pathway is to couple the CRISPR/Cas9 methodology with λ-RED oligonucleotide recombineering techniques. Ronda et al. (2016) combined the CRISPR/Cas9 system and λ-RED recombineering-based MAGE (Multiplex Automated Genome Engineering) technology referred to as CRMAGE. The technique proved highly efficient for performing genome engineering in *E. coli* with recombineering efficiencies between 96.5% and 99.7% for three genomic targets (0.68–5.4% for traditional recombineering techniques). CRMAGE is based on a two-plasmid system that requires the USER cloning technique to perform assemblage and rapid sgRNA exchange. The technique, when multiplexed, can be used to introduce two mutations in a single recombination step with nearly similar

efficiencies (Ronda et al. 2016). Based on a two-plasmid study of the CRISPR/Cas9 system by Jiang et al. (Jiang et al. 2013a; b), a triple plasmid study was performed applying a third plasmid containing gene for ParaB expressed λ-RED. The study showed an increase in the absolute mutant cells' percentage from 19% to 65% (Zhang et al. 2020).

23.5.2 Selection of Optimal Promoter for Cas9 and gRNA Expression

Choosing an optimal promoter for Cas9 protein and gRNAs expression could lead to the higher efficiency of genome editing. However, in previous studies, different promoters were chosen for Cas9 protein and gRNAs maintained in different vectors. Plasmids with low copies and constitutive promoters were commonly used for Cas9 protein expression to avoid negative effects on microbial growth that might result from Cas9 protein overexpression. On the other hand, the expression of gRNAs was usually performed by using high copy number plasmids under a strong promoter influence. The genes under the control of RNA polymerase III promoters were typically used for transcription of short gRNAs. However, RNA pol III promoters were found to be poorly defined, especially in industrially relevant filamentous fungi and yeasts, which was a major setback for the application of such promoters. A recent study by Nødvig et al. (2015) used a versatile CRISPR/Cas9 system containing gRNAs embedded in the middle of a large transcript transcribed by the enzyme RNA polymerase II. This large transcript with the gRNA in its middle was kept under the control of a strong constitutive promoter and terminator: *Aspergillus nidulans gpdA* promoter (*PgpdA*) and a *trpC* terminator (*TtrpC*), respectively. Two intrinsic ribosomal sequences, 5′-end hammerhead (HH) and 3′-end hepatitis delta virus (HDV), were used for gRNA flanking, supporting its release from the transcript and into the nucleus to initiate its function (Nødvig et al. 2015). Alternatively, the disadvantages of RNA pol III promoters could be addressed by using synthetic RNA polymerase III promoters. This approach was used in CRISPR/Cas9-mediated markerless gene disruption as well as integration in the oleaginous yeast, *Yarrowia lipolytica*. This microorganism is considered an important industrial strain due to its ability to carry out synthesis, modification, and storage of intracellular lipids such as omega-3 fatty acids and carotenoids. In this study, synthetic hybrid promoters prepared by combining native RNA polymerase III promoters with transfer RNA (tRNA) were used to transcribe sgRNA. The RNA pol III-tRNA hybrid promoter thus formed helped in the production of the mature and desired Cas9-targeting sgRNA molecule by exploiting the endogenous tRNA processing pathway. The series of promoters used in the study included (1) RNA polymerase II TEF promoter with Hammerhead ribozyme sequences and the HDV ribozyme sequences flanked at 5′- and 3′-ends of sgRNA molecule, respectively, and (ii) promoters of RNA polymerase III: RPR1, SCR1, and SNR52. The tRNA used to form the promoter-tRNA hybrid was glycine tRNA (tRNAGly). RNA pol III promoters were truncated by 16–25 base pairs downstream of RNA pol III binding sites. The truncated promoters were then combined with the glycine tRNA immediately upstream to

Table 23.2 Versatile models to address the challenges in CRISPR/Cas9 method

Challenges of CRISPR/Cas9 method	Disadvantages	Versatile models to address the challenges	References
Design and production of sgRNA	Dependence on protospacer adjacent motif of about 2–5 nucleotides located immediately downstream of target	Efficient generation of sgRNA can be conducted as the DNA target site recognition requires RNA-DNA pairing	Zhang et al. (2014); Nødvig et al. (2015)
	Both U3 snRNA and U6 snRNA are commonly expressed as housekeeping genes, hence cannot be employed to generate sgRNA	The sgRNA release from the transcript and its function can be supported by flanking it with two intrinsic ribosomal sequences	
Multiple mutations on target DNA site	Domination of NHEJ pathway over HR pathway for DNA repair in prokaryotes	Genetic and chemical modulations to divert the repair pathway from NHEJ pathway to HR pathway	Weninger et al. (2018)
Efficiency of genetic manipulation and off-target effects	Low genetic efficiency of gene editing efficiency of HR pathway	Combination of CRISPR/Cas9 with λ-RED oligonucleotide recombination technique to enhance the efficiency of HR pathway	Donohoue et al. (2018)
	Undesired mutations resulting from multiple highly similar or homologous sequences to the desired DNA sequences leading to off-target effects	Application of online software system controlling the expression of CRISPR/Cas9 components	

form the hybrids: RPR1'-tRNAGly, SCR1'-tRNAGly, and SNR2'-tRNAGly. These methods achieved 92% efficiency for single gene disruption, but the highest efficiencies were obtained for the SCR1'-tRNAGly hybrid promotor and the *Y. lipolytica* codon-optimized Cas9 expressed using the UAS1B8-TEF promoter (SCR1'-tRNAGly, 92 ± 5%; SNR52'-tRNAGly, 83 ± 6%; RPR1'-tRNAGly, 54 ± 7%; and Pol II TEF, 8 ± 2%). After co-transformation of homologous recombination donor plasmids and plasmids expressing Cas9 and sgRNA, a homologous recombination efficiency of more than 64% was observed, and disruption of the NHEJ pathway improved it to about 100% (Gao et al. 2016).

In addition to the described models, a number of other successful models have been designed and implemented in the experimental studies to effectively enhance the efficiency of CRISPR/Cas9-mediated genome editing. Some other versatile models for the same purpose include extending the incubation time under an appropriate selection pressure to allow the formation of DSBs until the mutagenic repair pathway functions (So et al. 2017), adopting appropriate binding sites for the sgRNA (Chuai et al. 2017), using Cas9 protein optimization codons (Bao et al. 2015, Table 23.2), etc.

23.6 Conclusion and Future Prospects

The recent emergence of CRISPR/Cas9 approach has its origins in defense mechanisms of various microorganisms, including bacteria and archaea. A greater inclination toward the biotechnological field has been achieved through CRISPR/Cas9-based precise gene editing, multi-gene editing, and gene regulation. The innovation achieved through the successful application of CRISPR/Cas9-based tools in industrially relevant microorganisms such as yeasts, bacteria, and filamentous fungi is one of their significant achievements. Compared to traditional marker-based tools for genetic engineering, the CRISPR/Cas9 system helped in rapid genetic editing of wild-type and industrial strains by enabling simultaneous editing of multiple genes in combination with marker cassette integration. The method provides a solid basis for high-throughput gene screening and single-stranded RNA editing techniques. However, additional considerations are required for proper implementation of this method in bacterial strains, as in most cases they already possess an endogenous CRISPR/Cas system which might crosstalk with exogenously introduced CRISPR/Cas components. More extensive experiments could be performed to include a broader range of industrially relevant microorganisms, such as the underrepresented *Pseudomonas putida*. Further studies need to be conducted to include thermophiles in CRISPR/Cas9-based studies by overcoming their applicability barriers, such as the need for thermostable Cas nucleases. High-throughput screening efficiencies that aid in the isolation and characterization of industrially important strains have also been provided by another technology, droplet-based microfluidics technology. So the combination of CRISPR/Cas9 method with droplet-based microfluidic technology may lead to greater flexibility in the field of industrial biotechnology in the future. Similar to other genetic engineering methods, the CRISPR/Cas9 method has advantages and disadvantages that need to be further explored. The limitations generally relate to off-site mutations, which are often lethal to host cells and the low efficiency and generation of the CRISPR/Cas9 component sgRNA and its delivery. Such limitations have been addressed through a variety of experimental studies that improve the effectiveness and efficiency of CRISPR/Cas9 system as a whole, expanding future doors for better implementation. The lower efficiency of the HR repair pathway needs to be addressed by more appropriate means to achieve satisfactory accuracy of CRISPR/Cas9 genome editing. In addition to the experimental prospects, the patent controversy on CRISPR/Cas9 applications needs to be steered in the right direction to remove the delay in adopting this method in various fields such as the pharmaceutical and biotechnology industries.

Acknowledgments The authors would like to thank the Department of Microbiology, Sikkim University, for providing the computational infrastructure and central library facilities for procuring references.

Conflict of Interest Statement The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Animal Cell Culture: Basics and Applications 24

Vikas Chandra, Ashutosh Tiwari, Kushal Kant Pant, and Renu Bhatt

Abstract

In 1855, German biologist Rudolf Virchow stated that every cell arises from the preexisting cell through division. A single plant or human cell can produce a whole organism or different cell types. The property of a cell by which it can give rise to all cell types by repeated division and differentiation and produce the entire organism is known as totipotency. In the animal world, this property of cells is utilized for different purposes, viz., gene therapy, recombinant DNA technology, tissue engineering, as well as for the synthesis of biologicals including enzymes, vaccines, monoclonal antibodies, growth factors, biopesticides, etc. Animal cell culture technology requires a special and sterile condition, to maintain cell culture, which helps in reducing microbial contamination. Different types of equipment such as biosafety cabinets, incubators, inverted microscopes, well plates, and different cell lines such as HeLa and Chinese hamster ovary (CHO) cell lines, etc., are routinely used in cell culture laboratories. Synthetic and natural culture media are used for culturing different types of cells based on their nutritional requirements. This chapter discusses the techniques, types of equipments, and cell lines involved in animal cell culture. Further, it describes how cultured animal cells can be used for various applications especially in human health and the biomedical field.

Keywords

Animal cell culture technology · Gene therapy · Tissue engineering · Recombinant DNA technology · Biologicals · Biosafety cabinets · Cell lines · Culture media · CO_2 incubator

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1_24

Abbreviations

μL	Microliter
3D	Three dimensional
AcMNPV	*Autographa californica* multiple nuclear polyhedrosis virus
ATCC	American Type Culture Collection
BHK	Baby hamster kidney
BMP	Bone morphogenic protein
BSL	Biosafety level
CHO	Chinese hamster ovary
DAPI	Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
ds-RNA	Double-stranded ribonucleic acid
EGF	Epidermal growth factor
EPO	Erythropoietin
FACS	Fluorescence-activated cell sorting
HAMA	Human anti-murine antibody
HAT	Hypoxanthine-aminopterin-thymidine
HeLa	Henrietta Lacks
HEPA	High-efficiency particulate air filter
HEPES	Hydroxyethylpiperazine ethane sulfonic acid
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HIV	Human immunodeficiency virus
HPACC	Health Protection Agency Culture Collection
HSCs	Hematopoietic stem cells
HuIFN-Beta	Human interferon-beta
IFN	Interferon
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IGF	Insulin-like growth factor
kDa	Kilodaltons
M	Minute
mAb	Monoclonal antibodies
moi	Multiplicity of infection
MOPS	Morpholino propanesulfonic acid
MRC-5	Medical Research Council cell strain 5
MS	Multiple sclerosis
NIH	National Institute of Health
PDGF	Platelet-derived growth factor
pfu	Plaque-forming-units
polyI:polyC	Polyinosinic:polycytidylic acid
SV40	Simian vacuolating virus 40
TGF	Transforming growth factor

t-PA	Tissue plasminogen activator
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WI	Wistar Institute

24.1 Introduction

Animal cell culture has matured to become an essential technology for many branches of the life sciences. The process by which the cells are removed from animal tissue or whole animal and grown under an artificially controlled environment in a favorable substrate with suitable nutrients and growth factors in vitro (in glass, broadly meaning in an artificial environment) is called animal cell culture (Jedrzejczak-Silicka 2017). When supplied with nutrients and growth factors, cells become capable of dividing and growing by mitosis (proliferation) till the availability of nutrients and space. These cells are selected for culture and maintained as independent units. The cell culture process allows and supports single cells to perform their vital functions. Each eukaryotic cell acts as a separate unit, just like any microorganism (Eagle 1955).

The animal cell culture has progressed from a simple, microscope-determined, observational science to a universally recognized technology and spread its roots as deep in the industry as in academia. Cell culture is very different from whole organ culture; organ culture means the upkeeping of entire organs or fragments of tissue, maintaining a balanced relationship between the associated cell types as found in vivo (Verma 2014).

Currently, specific cell culture is the ideal technique because of some degree of control over the conditions that allow consistency and reproducibility in cell growth and its study. However, the technique is referred to as "tissue culture," but this term could mislead to organ culture; therefore, "cell culture" is the most appropriate and logical term.

24.2 Biology of Cultured Cell

Cells that can be grown in culture are pervasive but include representatives of most major cell types. An important choice needs to be made when starting or establishing a cell culture according to the objective, scope of the project, and the nature of the experiments.

24.2.1 Primary Culture

Primary cell culture is established when the cells directly obtained from animal tissue are added to the growth medium and supplied with nutrients. The primary cultures are mostly based on embryonic tissue because of the superior growth potential and survival compared to adult tissue (Vazin and Freed 2010). Tissues are generally arranged in a highly ordered fashion and are comprised of different cell types; therefore, the objective of establishing a primary culture is to identify and select a single cell type from the ordered configuration of cells in a tissue (Heller and Fuchs 2015). In a primary culture when cells become confluent (cells occupied most of the available surface), they stop growing, and a new culture is established by inoculating some of the cells in a fresh medium, and the process is called subculturing or passaging (Masters and Stacey 2007). After this subculturing or passaging, the cells from the primary culture are established and referred to as secondary culture. Subsequently, many passaging cells are turned into a unique population (Valtink et al. 1999) that can continue growing through many subcultures and are termed as cell lines.

24.2.2 Cell Lines

Animal cell lines are vital and being used extensively for producing a wide array of therapeutic as well as prophylactic molecules, viz., hormone, cytokine, enzyme, interferon, antibody, vaccine, etc. As compared to animal models, cell lines can be handled very conveniently. The results obtained by experiments conducted using cell lines are highly reproducible. Moreover, cell lines are more efficient for the synthesis of biologicals at an industrial scale. Additionally, mammalian cell lines mostly secret biologically active protein molecules with appropriate folding patterns and accurate posttranslational modifications that are generally a limitation for bacterial and yeast systems. Some animal cell lines show adaptation for the growth in serum/protein-free media. This facilitates easy recovery of the biologicals that are secreted into the culture media. Moreover, the use of serum/protein-free media reduces the risk of contamination by viruses and mycoplasmas (Kaur and Dufour 2012).

24.2.3 Monolayer Cell Cultures

Anchorage-dependent cells usually require physical support to grow and are generally single-celled thick. They grow as a lawn of cells on the inner surface of the culture-ware (Harris et al. 2012).

24.2.4 Suspension Cell Cultures

The nonadhesive (to the surface of culture vessels) cells do not require any physical support or substratum and remain in suspension form in culture medium (Birch and Arathoon 1990).

24.2.5 Cell Line Repositories

The cultured cell lines that differ from each other on the basis of their origin (species and tissue type), as well as phenotype, can be procured from cell line repositories. One of the popular cell line repositories is the American Type Culture Collection (ATCC, website: www.atcc.org). Another very popular cell line repository is the Health Protection Agency Culture Collection (HPACC); the other name of this repository is the European Collection of Cell Cultures (website: www.hpacultures.org.uk). Such cell bank houses a large number of cell lines of different origins. Researchers can purchase those cell lines as per requirement.

24.2.6 Cell Types

In animal cell culture, cells are generally defined by the tissue from which they have obtained or derived and have characteristic morphology that can be observed and recognized through a light microscope. The selected cell type depends on the purpose of the study and research. There are five main types of animal tissues (Hatano et al. 2011) as described in Table 24.1.

24.3 Some Common Cell Lines Used in Laboratories

Some of the important cell lines commonly used in research and development laboratories are mentioned below.

24.3.1 Chinese Hamster Ovary (CHO) Cell Line

This cell line, popular for expressing human recombinant glycoproteins, contains a double-X chromosome (female origin) and exhibits an epithelial phenotype. It was originated from the animal Chinese hamster which is known scientifically as *Cricetulus griseus*. The CHO cell lines are well characterized, relatively stable, and transformed (naturally or due to transfection) into several other clones with different gene expressions. CHO cell lines are used in applications related to transfection, genetic studies, microfluidic design, and validation (Kim et al. 2012).

Table 24.1 Types of animal tissues with their description

Types of animal tissues	Description
Epithelial tissue	The tissues consist of layered cells that cover organs and line cavities and generally grow as a single cell monolayer in culture with a characteristic cobblestone appearance (Fuchs and Nowak 2008)
Connective tissue	The tissue contains fibroblasts which are bound to the fibrous protein collagen in the connective tissue and because of its excellent growth characteristics are the most widely used cells in the laboratory (Stopak and Harris 1982)
Muscle tissue	This tissue is made up of series of tubules (myotubes) that are developed from precursor cells (myoblasts), and they fuse to form multinucleate complexes with some structural proteins like actin and myosin (DiEdwardo et al. 1999)
Nervous tissue	It consists of neurons and supporting cells like glial cells. Since neurons are highly differentiated, it is not cultured widely. However, when the cells are supplied with nerve growth factors, they form cytoplasmic outgrowth called neuritis (Gordon et al. 2013)
Blood and lymph	These contain a variety of cells in suspension and include lymphoblasts that are extensively cultured for testing immune-regulating compounds (Hitchcock and Niklason 2008)

24.3.2 HeLa Cells

HeLa cell line is epithelial-like in morphology derived from cervical adenocarcinoma tissue removed from an Afro American lady Henrietta Lacks. HeLa cells are found susceptible to poliovirus as well as adenovirus type 3. Furthermore, HeLa cell line is suitable for expressing recombinant proteins, such as mouse metallothionein 1, human copper/zinc superoxide dismutase, hepatitis-B surface antigen, etc. The rapid growth and replication ability of HeLa cell line make it useful for producing a huge number of virus particles. Moreover, this cell line can also be used for testing antineoplastic activities of new drug candidates. A critical drawback of this cell line is that it may cross-contaminate other actively growing cell lines in its vicinity. However, this can be minimized by taking necessary precautions and care during the handling of the cell lines (Lucey et al. 2009).

24.3.3 NIH-3T3 Cell Line

This is an example of continuous cell lines. It is derived from fibroblasts isolated from NIH Swiss mouse embryos. NIH-3T3 cells are susceptible to sarcoma as well as leukemia viruses. NIH-3T3 cell line is a popular choice among researchers for gene transfection experiments. The cell line is also used extensively in microfluidic testing for adherent cells. A feeder layer made up of NIH-3T3 cells can be used for support and cultivation of other cells (Victorio et al. 2014).

24.3.4 Jurkat Cells

The Jurkat cells are T lymphocytic cells and were isolated from a 14-year-old boy suffering from acute T cell leukemia. This lymphoblast cell line grows in suspension culture with the expression of CD3 antigen on its surface. These cancer cell lines are relatively uniform in size. Jurkat cell undergoes either the caspase-8- or caspase-9-mediated apoptosis; hence it is suitable to be used as a positive control for apoptosis initiating as well as apoptosis inhibiting molecules (Abraham and Weiss 2004).

24.3.5 U-937 Cells

The U-937 cells express the CD95 (Fas) antigens. U-937 monocytic cells are originated from the blood cells of a lymphoma patient. These cells can grow in suspension culture and can be used for microfluidic experiments of cell separation and apoptosis studies (Passmore et al. 2001).

24.3.6 HT-29 Cell Line

This cell line originated from human colorectal adenocarcinoma tissue shows considerable variation in size and morphology and is highly aneuploid (68–72 chromosomes) in nature. HT-29 cell line is used for various cancer cell studies. Since it is a cancerous cell line, it must be handled carefully to avoid cross-contamination (Baricault et al. 1995).

24.3.7 Sf9 Cell Line

The Sf9 cells are originated from a pupal ovarian line of the insect fall armyworm (*Spodoptera frugiperda*). Sf9 cells are highly susceptible to infection with baculovirus and are able to proliferate at 26 °C. Moreover, these cells are able to secrete newly synthesized desired proteins. Such properties make them suitable for synthesis of recombinant protein molecules by using genetic engineered baculovirus expression vectors such as *Autographa californica multiple nuclear polyhedrosis virus* (AcMNPV)-based system. This cell line can be grown in serum-free media in a spinner flask or stirred tank (Yee et al. 2018).

24.3.8 COS1/COS7 Cells

The COS1/COS7 cells are fibroblast-like, African green monkey kidney cells obtained from CV-1 simian cell lines transformed with defective mutants of SV40 encoding wild-type T antigen. These transformed cells contain T antigens and can allow the multiplication of recombinant SV40. COS cells are useful for the synthesis

of recombinant protein molecules, viz., HSV-1 glycoprotein B, ricin B chain, von Willebrand factor, thrombomodulin, CD7, etc. (Hancock 1992).

24.3.9 Hybridomas

Production of monoclonal antibodies (mAb) by Kohler and Milstein with immortalized cells secreting specific antibodies (Köhler and Milstein 1976) has led to the development of hybridomas (hybrid cells resulting from the fusion of immortal myeloma cells with antibody-producing plasma cells). Hybridomas are either maintained in suspension culture inside roller bottles or stirred bioreactors or immobilized with suitable matrices, viz., microbeads and microcapsules (Tomita and Tsumoto 2011). Many monoclonal antibodies produced by hybridoma cells are licensed or are under clinical trials for biomedical purposes. Although the majority of mAbs that have been obtained are of mouse and rat origin (Berger et al. 2002), human mAbs can be obtained by immortalizing lymphocytic cells exposed to a specific antigen. This includes antibodies for HIV-1 envelope glycoprotein, cytomegalovirus, and rubella, etc. (Newsome and Ernstoff 2008).

24.4 Cell Culture Media

The culture medium is the most crucial factor for the cultivation of cells in an artificial environment. Culture media is an appropriately designed mixture of nutrients, growth factors, amino acids, salts, and other vital supplements that closely resemble their in vivo compositions (Yao and Asayama 2017). The animal cell culture media essentially have ten necessary components which are described in the following sections.

24.4.1 Media Components

The components are listed in Table 24.2.

The complete and exact formulation of animal cell culture media is either very complicated or not known completely; therefore, the initial media used for the cultivation of animal cells were heavily dependent on the incorporation of biological fluids like the serum, plasma, lymph, embryo extract, etc. The most suitable media for any cell culture needs to be determined experimentally since the different cells have diverse nutritional requirements at various stages of growth (Verma et al. 2020).

Cell culture media can be categorized into two groups as described in the following sections.

Table 24.2 Components of media for cultivation of animal cells in vitro

Media components	Examples
Inorganic salts	Inorganic salts of calcium, magnesium, sodium, potassium etc.
Nitrogen sources	Amino acids (essential and nonessential)
Vitamins	Vitamin B12 and other vitamins (water-soluble and fat-soluble)
Growth factors and hormones	PDGF, VDGF, EGF, VEGF, IGF
Energy source	Glucose and fructose (monosaccharides); glutamine (amino acid)
Fat and fat-soluble components	Fatty acids, glycerol, cholesterol
Antibiotics	Antibacterial (penicillin, streptomycin) and antifungal (amphotericin B) compounds
pH and buffering systems	HEPES, sodium bicarbonate, CO_2-carbonate system
Nucleic acid precursors	Purines and pyrimidines, phosphate sugars
Gases	CO_2 levels are maintained at the desired percentage in CO_2 incubators

24.4.2 Natural Media

As its name suggests, it consists of naturally occurring biological fluids, such as plasma and embryo extract, which support the growth and divisions of animal cells. Natural media can be further classified into the following three types:

1. **Biological Fluids**: Serum, lymph, fetal calf serum, amniotic fluids, etc., fall under this category and are generally used as media after rigorous checking for any contamination or toxicity.
2. **Coagulants or clots**: Generally, plasma is separated from the blood of chicken, bovine, or human and used as media.
3. **Tissue extract**: Various tissue extracts from chicken embryos, liver, spleen, and bone marrow are available commercially and used as media for animal cell culture.

24.4.3 Artificial/Synthetic Media

The synthetic media is prepared artificially and contains a basal medium that may be supplemented with serum, growth factors, or other suitable organic compounds (Morgan et al. 1950). The artificial media is categorized into the following groups.

1. **Serum-free media**: Supplemented with crude fractions of protein with basal salt solution (Barnes and Sato 1980).
2. **Serum containing media**: Bovine, human, and other sera are generally incorporated for supplementation.

3. **Protein-free media**: Amino acids and hydrolysates of proteins are incorporated for supplementation.
4. **Xeno-free media**: Only human-sourced components are incorporated for supplementation.
5. **Chemically defined media**: Medium with pure organic as well as inorganic components and recombinant proteins as supplements (Arora 2020).

24.5 Laboratory Design and Layout

Animal cell culture is very sensitive to any kind of contamination, that is why the cell culture laboratory needs to be designed scientifically to avoid various contamination and for the smooth operation of the laboratory. For the cultivation of animal cells, the laboratory must essentially allow the handling of cultured cells under an aseptic environment with the least possibility of contamination (Wesselschmidt and Schwartz 2011). This becomes crucial because the rate of proliferation of cells of bacteria and fungi is very high compared to animal cells. Contaminations caused by bacteria or fungi lead to significant depletion of nutritional constituents of media due to the very fast cell division of microbial cells. However, contamination by bacteria and fungi can be eliminated or reduced by following good laboratory practices and procedures carefully, using antibiotics or antifungal agents, and, most importantly, by proper sterilization of the components (Clarke et al. 1989). It is very difficult to protect the cultivated cells from mycoplasma contamination because the majority of the antibacterial agents target the cell wall that is not present in mycoplasma. Contamination caused by bacteria can be easily detected at its later stages and verified by microscopic observation. Additionally, cell culture media show the change in color due to a drop in pH by bacterial contaminants through pH indicators, e.g., phenol red. Fungi contaminating the cultivated cells can be identified by observing fungal bodies through the microscope (Stacey 2011).

The purpose of designing a cell culture laboratory is to eliminate or at least reduce the level of contaminants as much as possible for a skilled experimenter. Alexis Carrel was inspired by the methods and practices performed to maintain an aseptic environment during surgery in hospital operation theaters, and he borrowed some methods in animal cell culture technique (Geraghty et al. 2014).

While designing an animal cell culture laboratory, one should keep in mind that areas for washup, sterilization, and sterile handling of biological samples must be physically separated from each other. If possible, the animal cell culture laboratory should be separated from the preparation, sterilization, and washup areas. In general, cell culture facilities required for the cultivation of animal cells must be located in a small room with minimal movement of personnel.

High-efficiency particulate air (HEPA) filters or electronic filters must be preferably used to filter air incoming into the laboratory. This kind of setup may also generate positive pressure inside the facility to minimize the airborne contaminants. Cells must be handled within laminar flow cabinets that maintain an aseptic environment due to the presence of HEPA filters in them (Coté 2001).

Establishing an animal cell culture laboratory requires sincere efforts by dedicated personnel. The cell culture laboratory must house a few equipments and supplies essential for cell cultivation job under an aseptic environment. Some of the important equipments may be classified as either sterile culture based and include laminar biosafety hoods, incubators, and sterilizers or analysis/preparatory based that includes equipments, used to handle and process samples, such as centrifuges and culture wares, as well as personal protective equipment (Phelan and May 2016).

24.6 Essential Equipment Required in Animal Cell Culture Laboratory

24.6.1 Biosafety/Laminar Flow Cabinets

The biosafety cabinet is crucial for handling cultivated cells aseptically. It also ensures the protection of operators as well as biospecimens/cells. On the basis of biosafety levels (BSL), the cabinets are categorized into various classes. General-purpose works like preparation of culture medium or experiments with non-primate cells may be performed in small front opening cabinets that are inexpensive. Biosafety cabinets are furnished with an ultraviolet lamp to sterilize the working surface before and after handling biospecimens/cells. The surface of the cabinet needs to be disinfected regularly, and bottles or flasks should not be stored in the cabinet. Lack of contaminants indicates the practice of good aseptic techniques. However, simple cabinets do not eliminate the risk of exposure of the operator to possible pathogens. Handing cells originated from humans and other primates may increase the risk of transmission of pathogenic microbes. To eliminate the risk of possible infection, most animal cell culture laboratory uses open-fronted biosafety cabinets where only experimenter's arm enters the aseptic area. Such safety cabinets offer a space supplied with an upright flow of filtered air and a plane working surface where disinfection can be done easily as and when required (Herman and Pauwels 2014).

Class II biosafety cabinets are suitable for experiments involving agents exhibiting a low to moderate level of toxicity or infectivity. After passing through HEPA filter, air gets sterilized and offers personnel protection against unsafe agents. A sterile space for culture handling is formed by recirculating a large percentage of air (70–80%) to create an air curtain with a typical flow rate of 0.4 m/s. Air exhausted through the process is allowed to pass through HEPA filters in order to protect the surroundings from potentially pathogenic microbes or toxic chemicals (Sewell 1995). Class II biosafety cabinets are designed in such a way so the operator's hand can freely access the working area but the operator's breathing is prevented to reach the working area by a transparent cover. HEPA filters made up of continuous sheets of submicron glass fibers that fold back and forth over a corrugated spacer are designed in such a way so that they can retain airborne particles and aerosols. A standard class II cabinet HEPA filter ensures 99.99% efficiency in entrapping 0.3 μm particles. A UV lamp is also present in a class II biosafety cabinet, and it maintains a

sterile environment inside the cabinet while not in use. Generally, the horizontal working area of biosafety cabinets is cleaned by spraying and wiping with 70% ethanol.

The class III biosafety cabinet is an enclosed and isolated hood found in some special laboratories specifically designated for handling highly pathogenic agents. The cabinet is fully sealed and has gloved pockets to allow manipulation of cultures. In this system, two or more HEPA filters are used so that all pathogenic microbes are removed from exhausted air. Equipment used in the cabinets are passed through airlocks and removed directly to autoclaves (Kin et al. 2007).

24.6.2 Incubators

An incubator's principal function in animal cell culture is to maintain homeostasis by primarily controlling the temperature of the cultures and samples. Incubators are an essential requirement for providing regulated temperatures for the cultivation of cells. The incubator itself is usually a sterile environment, and the culture-wares further obstruct the entrance of microbes. Laboratory incubators may differ by architecture, yet each one of them characteristically allows adjustment of temperature as well as air composition in a controlled fashion. Water jacket incubators are excellent in exhibiting thermostability. Most mammalian cell culture incubators, in order to provide consistent culture conditions, run at 37 °C with 90% relative humidity and 5% CO_2 content. A minor reduction in temperature compared to optimum value can decelerate the cellular growth and proliferation rate, while a rise in temperature causes greater damage to cultivated cells. The cell hardly survives for a few hours at 39 °C. Cells quickly expire beyond temperatures of 40 °C. Humidity, reduced by the loss of water from medium through evaporation, can be maintained by the humidified atmosphere for open culture systems such as Petri dishes and multi-well plates. Humidity in a higher-end system is regulated by steam generators, while in regular laboratory incubators it is maintained by putting a tray filled with water at the bottom surface inside incubators. The atmosphere inside the incubator is supplied with 5% CO_2 for maintaining the pH of media containing carbonate buffer. This CO_2-enriched atmosphere of the chamber serves as a buffering system that involves equilibrium with bicarbonate present in media used for the cultivation of cells. The required percentage of CO_2 for maintaining the pH of culture media at approximately 6.9–7.4 depends upon the amount of bicarbonate per milliliter of media. Irregular CO_2 concentration inside incubators is manifested as a change in the pH and hence the color of the media. Low CO_2 levels lead to an increase in pH, while high CO_2 levels lead to a decrease in pH. This bicarbonate-CO_2 buffer system is excellent to maintain a nearly constant pH in the medium, but the major disadvantage of this is that a rapid increase in pH of media to alkalinity is observed when the culture vessel is taken out of the incubator.

pH of the media can be maintained without a high carbon dioxide environment by use of some organic buffers like HEPES (hydroxyethylpiperazine ethane sulfonic acid) and MOPS (morpholino propanesulfonic acid) (Eagle 1971). To achieve a high

degree of pH regulation, organic buffers are used in combination with a bicarbonate-CO_2 buffering system.

The CO_2 incubators with fixed CO_2 tension and humid environment have become an indispensable tool for the cultivation of cells in vitro. A single incubator can be used for multiple cell lines, and to avoid cross-contamination, they must be kept in a designated portion inside the incubator (Ham and Puck 1962).

24.6.3 Autopipettors

For routinely maintaining cells in culture or preparation of cells for various assays, handling of the sample in a sterile manner is essential to preserve viability and functionality. To achieve this, the handling of cells is performed inside the laminar flow hood. Suspension culture must be handled by the use of serological pipettes fitted in autopipettors. The pouring technique must not be followed for suspension cultures of animal cells. Generally, sterile disposable tips fitted in pipettors must be used for handling small volumes. This is required to maintain the sterility of cells as well as reagents. Serological pipettes are recommended for volumes larger than 1 mL, and they should not be operated using the mouth or even suction bulb. To overcome this, an automatic pipettor, with a 0.22 μm barrier filter that prevents unintentional aspiration of media into the pipettor, must be used. This practice also reduces the risk of cross-contamination.

24.6.4 Culture Vessels

The culture vessel for the growth and maintenance of cells in vitro must be selected on the basis of the scale of operation and nature of cells required. Culture volumes can range from 100 μL to 10,000 L with existing commercial equipment. In smaller volume vessels, pH and oxygen concentration may shift in the course of cellular proliferation. However, these vessels can easily be inserted into and taken out of the laboratory incubators. They also provide a flat bottom for adherence of cells to the substratum.

24.6.4.1 Multi-Well Plates

The main advantage of using such vessels is their ability to accommodate samples in replicates. They are made up of tissue culture-grade polystyrene. These multi-well plates are available commercially in different numbers of wells per plate, viz., 6-, 12-, 24-, 48-, and 96-well plates. The purpose of the different number of wells in a multi-well plate is to facilitate these plates for various applications and purposes. For example, a 24-well plate can accommodate a volume of 3 mL per well and is convenient for conducting toxicity and stimulatory assays. Similarly, a 96-well plate is suitable for cloning and replicate assays, as it holds a volume of 0.3 mL per well, and it can be read by a 96-well plate reader to enable automation.

24.6.4.2 T-flask

The T-flask or tissue culture flask is useful for the cultivation of both adherents as well as non-adherent cells. It can hold a volume of 2–100 mL. T-flasks come in several dimensions based on the area of the bottom surface that is accessible for attachment of cells to it (Table 24.3). The angular necked design of these flasks makes sterile manipulations easy. In order to maintain the equilibrium of gaseous phases inside and outside T-flasks during incubation of cultivated cells, the caps of these flasks must not be fitted tightly.

24.6.4.3 Spinner Bottles

These vessels are best for the cultivation of non-adherent cells. It is made up of glass and contains a Teflon paddle in its center. It also contains a magnet that helps in agitating the media when the spinner bottle is kept over a magnetic stirrer where the rotation speed ranges from 10 to 300 rpm can be achieved.

24.6.4.4 Roller Bottle

These cylindrical-shaped vessels provide a greater surface area for the cultivation of cells and are available in three standard sizes with growth surfaces of 490, 850, or 1750 cm^2. They can be filled with 250–500 mL of medium. These vessels are made up of plastic and are best for the cultivation of adherent cells.

24.6.5 Microscopes

Modern microscopes provide the very high resolution required for studying cells. Many microscopic techniques have been devised to perform cellular analysis. There are mainly two types of microscopes used in cell monitoring. The inverted microscope is very crucial for examining cells in culture to monitor their health and growth, and a standard microscope is required to count cells in a culture sample into the counting chamber. For some special needs, other microscopes such as fluorescence and confocal microscopes are also used in animal cell culture laboratories.

The basis of fluorescence microscopy is the excitation of the specimen with a specific wavelength of light and observing the emission of fluorescent light by the specimen. Epifluorescence microscopy is a very common fluorescence microscopy technique. This employs an objective lens that also acts as a condenser so that the specimen can be illuminated from above. The high-energy small wavelength light

Table 24.3 T-flasks used in animal cell culture laboratories

Type of T flask	Surface area for cell attachment (cm^2)
T-25	25
T-75	75
T-150	150
T-175	175

(excitation light) excites fluorochromes like acridine orange, diamidino-2-phenylindole (DAPI), and the fluorochromes absorb light and fluoresce brightly.

A confocal microscope provides high-resolution images of cells when it is used in animal cell culture laboratories. Basically, it is a fluorescence microscope, so the materials or cells which are to be visualized must be stained with fluorescent probes. It is also known as a widefield fluorescence microscope. Confocal microscopes are prominently used for cellular and histological studies. Spinning disk confocal microscope, a type of confocal microscope, is used for live cell imaging, which allows scientists or researchers to visualize live cells or cell lines without affecting their stability and physiology (Ettinger and Wittmann 2014). Three-dimensional (3D) cell cultures are thick, and they do not allow lights to pass through them. Thus, the microscopes which are used to visualize thin and transparent cultures fail to visualize three-dimensional cell cultures. Due to its penetration to relatively higher depth, confocal microscopes can be utilized for high-resolution imaging of three-dimensional cell cultures (Graf and Boppart 2010). Confocal microscopes are also used in the morphological observation of cell populations in tissue engineering in cancer researches (Goliwas et al. 2017).

24.6.6 Centrifuges

Centrifuges are another essential equipment to harvest cells from culture; for most cells a centrifugal force of 150–200 $\times$ *g* for 5–10 m is adequate to segregate cells from the media. Rotors of centrifuge system are designed either as fixed angle or swing-out buckets. Higher centrifugal drive or performing centrifugation for a very long duration causes injury to cells by tamping them at the base of the vessel. For routine centrifugation, cell suspensions are kept in centrifugation tubes generally made up of plastic materials and can hold the liquid up to either 50 or 15 mL. These tubes are placed in swing-out buckets fitted inside bench centrifugation machines. Small-volume cultures/samples must be centrifuged in benchtop microcentrifuges.

24.6.7 Liquid Nitrogen Reservoir

Cells are found stable almost indefinitely when cryovial tubes, containing a suspension of the cells in cryoprotectant media, are kept in liquid nitrogen (−196 °C). The cells suspension (approximately 1 million cells/mL of freezing medium) must be stored in cryovials (generally having the capacity of holding 2 mL liquid). The freezing media contain 10% glycerol or dimethyl sulfoxide (DMSO) as a cryoprotectant that prevents cellular damage in the course of the freeze-thaw cycle. Typically freezing media is supplemented with a growth medium or serum. Nevertheless, it is very essential to practice the right procedure of freezing as well as thawing to sustain the viability of the preserved cells. The thumb rule of "freeze slowly and thaw quickly" must be followed for achieving a higher percentage of viable cells during the freeze-thaw cycle.

24.6.8 Other Types of Equipment

Apart from the above lists, a standard cell culture laboratory also contains several other types of equipment/instruments. Hemocytometer is used for counting cells under a microscope. It is also used for discriminating and counting live and dead cells by staining with trypan blue dye (dye exclusion test). Refrigerators (0–4 °C, −20 °C, and −80 °C) are used for storage of thermo-labile chemicals. −80 °C freezer and liquid nitrogen cans are used for cryopreservation of cells. Water bath incubators are used for thawing cryopreserved cells and incubating cells during various assays that demand specified temperatures. Flow cytometer and fluorescence-activated cell sorting (FACS) machines are routinely used in advanced cell culture laboratories for counting and/or sorting cells and determining the characteristics and function of specific cell types, especially by immunophenotyping. They are also used for the detection of biomarkers on the cell surface, cell cycle analysis, cellular proliferation assay, apoptosis assay, diagnosis of disease based on cellular abnormalities, etc.

24.7 Applications of Animal Cell Culture

The technique of cultivating animal cells in vitro plays a significant role in synthesizing a broad range of compounds that can be extracted and purified as products from the cultures. Many of these compounds have good value as healthcare products. Major challenges with these products are isolation and purification of the product at an acceptable purity level. To obtain cell culture products in an adequate amount, a scale-up of the cell culture is needed. The last 2–3 decades have witnessed major innovations and developments of mammalian cell culture techniques for the production of biologicals. It provides an indispensable bio-device for the manufacture of several biomaterials and even tissues and organs.

Some of the major applications of technology of cultivating animal cells are enlisted in Fig. 24.1 and described in detail in the following sections.

24.7.1 Viral Vaccines

Polio vaccine was the first vaccine produced at a large scale by cell culture techniques. The invention of the polio vaccine ultimately led to the development of animal cell culture techniques for the manufacture of a large number of viral vaccines for a variety of diseases. Production of the vaccine in animal cell culture offers a consistent and efficient method of manufacturing compared to alternative methods. The vaccine (here viral antigen in the nonpathogenic form), when injected in the recipient, induces the formation of the corresponding antibody to provide some protection against the live virus and associated disease. Generally, an inactivated pathogenic virus that has been inactivated chemically or an attenuated

Fig. 24.1 Major applications of animal cell culture

live virus is used as a vaccine. Live attenuated viral vaccines are preferred because they can be administered in small but effective doses (Rodrigues et al. 2015).

The major concern while choosing a cell line for vaccine production is safety. Due to the risks associated with tumorigenic cell lines such as HeLa, the use of such cell lines as a substrate for viral propagation was rejected, and in 1954 regulations were made to produce vaccines for humans from primary cells derived from healthy monkeys. The polio vaccine was produced by viral propagation on primary monkey kidney cells, but later it was found infected with a newly discovered tumorigenic virus SV40. The extensive study and characterization of normal human diploid fibroblasts have led to their acceptance for vaccine production. WI-38 and MRC-5 cells have played a significant role in the manufacturing of polio vaccines. Although these cells have a finite lifespan, their use in large-scale propagation is much easier. In this chain Vero (African green monkey cells) was the first continuous cell line accepted for human vaccine production (Jordan and Sandig 2014).

24.7.2 Propagation of Viruses

Animal cell cultures are excellent in the propagation of viruses. The viruses can be propagated in very high density by inoculating them into the culture of cells. Type of the cell to be used in viral propagation should be susceptible to the particular virus. Anchorage-dependent cells are found well suitable for large-scale production and viral growth. Viruses follow a lytic life cycle in a cell culture system. The lytic cycle consists of four important and distinct phases, namely, adsorption, penetration, replication, and release. In the lytic life, cycle virus employs cellular machinery to synthesize new virus particles, and this ultimately led to cell lysis and release of new viral particles.

The quantity of the virus is expressed in plaque-forming units (pfu) that corresponds to the formation of plaques on a monolayer of cells. The viral production can be done by adding the virus to cell culture at a multiplicity of infection (moi) of 01–10 pfu/cell and that can be expected to increase up to 10^3–10^4 pfu/cell in a period of propagation (Warnock et al. 2006).

24.7.3 Monoclonal Antibodies

Monoclonal antibodies are highly specific. They have paratops (antigen-binding site) specific to a single type of antigen epitope. They are produced using hybridoma technology. This technology uses B lymphocytes fused with myeloma cells (tumor cells), and due to this, they can grow indefinitely in vitro (Yokoyama et al. 2013). This revolutionary technology was discovered by two prominent scientists Cesar Milstein and Georges Kohler in 1975. Both the scientists shared the 1984 Noble Prize for physiology and medicine with Niels Jerne (Bretton et al. 1994). This technology is also of utmost importance as it has opened new gates toward different approaches used in diagnosing, treating, and preventing diseases (Pandey 2010).

For the production of hybridomas, the B lymphocytes are obtained from the spleen of the animals (e.g., mice). The antigen against which the antibody is to be produced is injected into the animal. As each B cell is programmed to respond to a specific and single epitope or antigenic determinant site, the B lymphocytes specific to the antigen injected are activated. The animal is then sacrificed, and the lymphocytes are extracted from the spleen cells. These cells are then made to fuse with the myeloma cell lacking HGPRT (hypoxanthine-guanine phosphoribosyltransferase) gene. Cells are screened for successful fusion using the HAT (hypoxanthine-aminopterin-thymidine) medium (Parray et al. 2020). Nowadays, the Chinese hamster ovary (CHO) cell line, PER.C6 human cell line, and NS0 murine myeloma cell line have shown their promising role in industries for large-scale manufacture of mAbs (Li et al. 2010).

Monoclonal antibodies are very specific to bind with an antigen and have been used as therapeutic agents particularly in targeted drug delivery. OKT3 developed by Ortho Pharmaceuticals was the first murine mAb to be licensed for therapeutic applications. This recognizes a specific surface antigen (CD3) on T lymphocytes

and is very effective in preventing immunological rejection of transplanted kidneys. Many monoclonal antibodies have been developed to target membrane-bound tumor-specific antigens. The antibodies are designed in a configuration that can cause destruction of the targeted tumor cells. Conjugating antibodies with molecules exhibiting radioactivity or toxicity maintain localized high concentrations of such cytotoxic agents inside tumor tissue resulting in cytotoxicity to the target cells. One of the limitations of using murine antibodies for therapeutic purposes is the development of unwanted human anti-murine antibody (HAMA) immune responses. But the development of human chimeric antibodies soon undertook the market of therapeutic monoclonal antibodies (Heinrich et al. 2003). These humanized antibodies lack the HAMA effect and remain stable for a longer duration than murine antibodies (less than 20 h).

24.7.4 Therapeutic Proteins

Proteins obtained from biological sources are very important for substitution therapy. However, the extraction requires a large quantity of tissue, blood, or urine to get a sufficient amount of each product. This problem was solved by extracting proteins from nonhuman sources. But this can elicit an adverse immune response in human recipients. The major risk associated with these extractions was the danger of contamination from infectious agents such as HIV and prions. But now most of these natural products can be produced in large quantities by isolating and expressing specific genes in a host cell line (Zhu et al. 2017).

Therapeutic proteins that can be produced using a cell culture system are enlisted in Fig. 24.2 and explained elaborately in the following sections.

24.7.4.1 Interferons

A compound that interferes with viral propagation in cells in culture has been shown by Isaacs and Lindenmann and was termed interferon. They have also shown protection of noninfected cells from subsequent infection by culture medium that previously supported the growth of viruses. This led to the discovery of a group of a protein termed interferon (Isaacs and Lindenmann 1957). Interferon can be referred to as an inducible secretory protein produced by eukaryotic cells in response to viral infection or other stimuli. Now the interferons are recognized as a family of cytokines with antiproliferative, antiviral, and immunoregulatory properties. The therapeutic value of interferon lies in their antiviral activity and ability to retard the growth of tumor cells, and there are a number of viral infections and cancers that can be treated effectively by interferons (Samuel 2001). Generally, interferons are a group of comparatively small proteins that contain 140–170 amino acids and can be classified into three major types IFN-α, IFN-β, and IFN-γ (Bandurska et al. 2014).

IFN-α

The IFN-alpha is predominantly secreted by B lymphocytes. Isolation of IFN-alpha from leukocytes was very popular in the 1960s, but the yield from this process failed

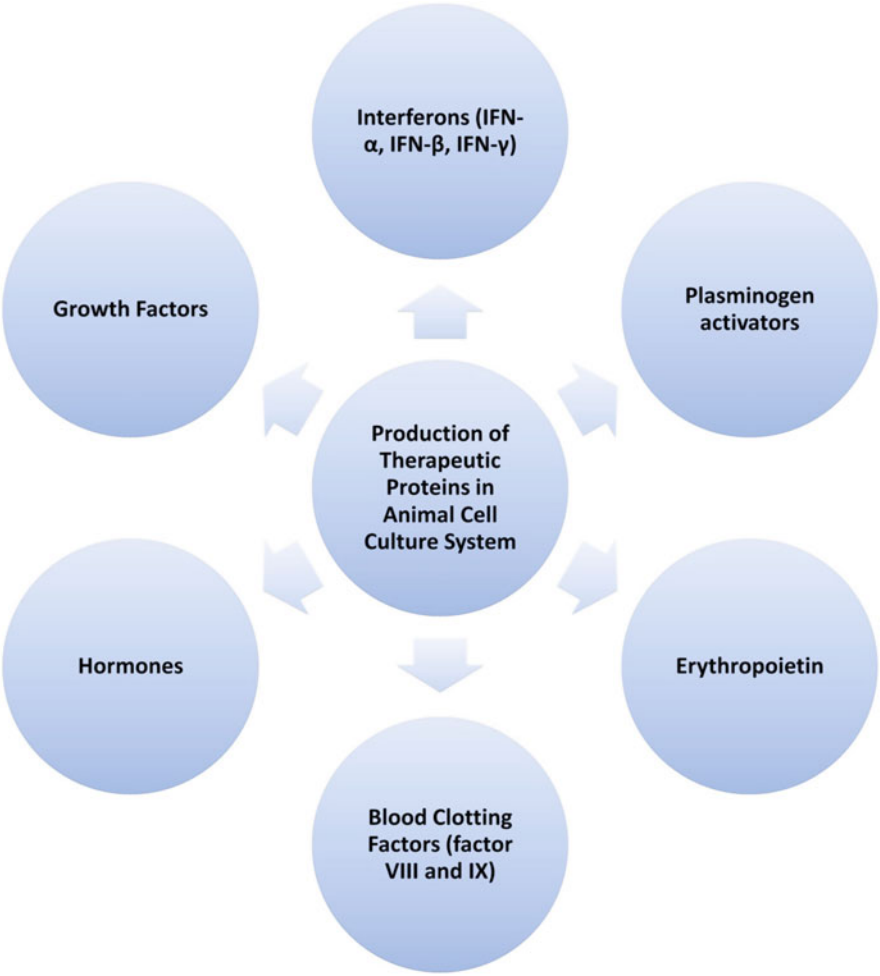

Fig. 24.2 Therapeutic proteins that are produced commercially in the cell culture system

to fulfill the growing need of the interferon market. A large-scale cell culture production system (10,000 L capacity) was developed by Wellcome Trust (UK) in batch fermenters and human B lymphoblastoid cell line (Namalwa) after induction with Sendai virus was used. The cells are well adapted to grow on low-cost serum-free medium, and in fact, IFN-alpha was the first product from a tumor cell line to be licensed for human therapy (Kontsek 1994).

IFN-β

The fibroblasts predominantly secrete IFN-beta. IFN-beta and IFN-alpha-2 are the only naturally glycosylated human interferons. Human interferon-beta (HuIFN-beta) is 25 kDa glycoprotein with 166 amino acid residues, while non-glycosylated form is

17.5 kDa (Karpusas et al. 1998). HuIFN-beta is produced by fibroblast cells in response to viral stimulation or ds-RNA encounter. The antiviral and antiproliferative properties of HuIFN-beta have been used in the treatment of certain type of tumors. The large-scale production of HuIFN-beta was obtained from cultures of human foreskin fibroblasts by superinduction process, which generally involves the introduction of synthetic polynucleotide (polyI:polyC) that may be followed by a regime of antibiotic supplementation to boost interferon synthesis (Clark and Hirtenstein 1981). The recombinant HuIFN-beta was approved in 1993 for the treatment of multiple sclerosis (MS). HuIFN-β1b, commercially marketed as Betaseron, Avonex, and Rebif, is available for MS treatment.

IFN-γ

The natural IFN-gamma is predominantly synthesized by T lymphocytes, and this could be stimulated by a variety of antigens or mitogens. In cell culture, generally, staphylococcal enterotoxin is employed as an inducer to stimulate the extracellular release of IFN-gamma. Commercial production of IFN-gamma is achieved by recombinant mammalian cells transfected with isolated genes (Savan et al. 2009).

24.7.4.2 Plasminogen Activators

In the western world thrombosis is one of the main reasons for premature death. In thrombosis, fibrin is deposited in the circulatory system and causes blockage in blood flow. Under healthy conditions, fibrin formation is controlled via a clotting cascade which is triggered during wound healing. Tissue plasminogen activator (t-PA) plays significant role in solubilizing insoluble fibrin to its soluble products. The large-scale production process was developed for tissue plasminogen activator (t-PA) by Genetech and involves the use of transfected CHO-K1 cells (Anderson et al. 1991).

24.7.4.3 Blood Clotting Factors

Hemophilia is characterized by an inactive clotting cascade due to failed formation of fibrin. The clotting factors VIII and IX are generally absent in hemophilia A and hemophilia B, respectively.

Factor VIII

The factor VIII is a large complex glycoprotein of 265 kDa encoded by 186-kb-long gene with 26 exons and 25 introns (Lawn and Vehar 1986). It was produced in vitro by transfecting mammalian kidney cell line (BHK) with an expression vector. The secreted recombinant factor VIII is generally stabilized by adding the von Willebrand factor. The manufacture of factor VIII at the industrial level is done in BHK cells containing a high copy number of the factor VIII gene (up to 150/cell) (Orlova et al. 2013).

Factor IX (Christmas Factor)

Factor IX is a glycoprotein of 57 kDa secreted by hepatocytes in vivo. The production of factor IX requires glycosylation and γ-carboxylation to achieve the full

activity; therefore, a rat hepatoma cell line was used to express the factor IX gene in vitro (Anson et al. 1985).

24.7.4.4 Erythropoietin

The erythropoietin (EPO) is a glycoprotein hormone of 30–35 kDa. The polypeptide chain of this protein consists of 165 amino acids. EPO, synthesized by the kidneys, ensures continuous RBC production in the bone marrow (erythropoiesis). Kidney failure leads to a low level or absence of EPO in the bloodstream which leads to the impairment of RBC production and ultimately anemia. However, the condition is curable by the administration of exogenous EPO. The carbohydrate component is very crucial for complete activity as partially glycosylated EPO is clinically less effective. EPO is commercially produced in vitro by transfecting CHO cell lines with an expression vector containing EPO gene, and the resulting recombinant EPO is effective against chronic kidney failure-associated anemia (Inoue et al. 1995).

24.7.4.5 Growth Factors

These are the proteins responsible for cell proliferation and differentiation. Common growth factors include EGF, TGF, PDGF, and insulin-like growth factors, etc., which are available in the market. Commercially Osigraft/Eptotermin alpha (bone morphogenic protein, BMP) and InductOS/Dibotermin (BMP) are available for tibia fractures and spinal surgery. Their production involves CHO cell lines (Hunt et al. 1997).

24.7.4.6 Hormones

Hormones (such as insulin, glucagon, growth hormone, etc.) regulate physiological functions and can be used for therapeutic purposes as well. Insulin and growth hormone are the first biopharmaceuticals to be approved for therapeutic purposes. However, they were produced in microbial cells. Gonadotropic hormones are available commercially for treating female infertility. CHO cell line can be used for its production (Ghavim et al. 2017).

24.7.5 Biopesticides

Biopesticides (biological agents that control agricultural pests) are environmentally safe and provide effective control over insect and plant disease and are gaining more and more acceptance. Baculoviruses are used worldwide against caterpillars as a biocontrol agent. Cell lines such as Sf21 and Sf9 are commonly used for biopesticide production (Chandler et al. 2011).

24.7.6 Gene Therapy

Gene therapy is a practice involving the manipulation of genes to improve the quality of life of a diseased person. Gene therapy is a very promising approach,

regardless of the potential technical challenges faced during the transfer of genetic material. Advancement of gene therapy could not have been possible without employing the techniques of cultivation of animal cells. Primary targets for gene therapy are diseases like cystic fibrosis, hemophilia, and muscular dystrophy. These are examples of monogenic diseases generally caused by defects in a single gene. Gene therapy is a step-by-step process in which the first step is the identification of disease-causing genes. Faulty genes are isolated after their identification. Finally, correct expression construct is generated that may replace the disease-causing gene. Finally, the integration of the correct version of the gene is achieved by delivering the correct expression construct. In the in vivo approach, the correct gene construct is inserted directly at a specified location of the recipient's body.

In the ex vivo approach, cells are first isolated and then treated with the correct genetic construct, are proliferated, and are transferred back to the recipient's body. The first approved product for the gene therapy was Gendicine developed by a Chinese firm. The p53 gene was expressed in recombinant adenovirus, and SBN cell was transfected for the production of Gendicine (Wilson 2005).

24.7.7 Tissue Engineering

The modern development in cell culture technologies has enabled us to reconstitute human tissues from combinations of cell types that can be grown in culture. The most successful application of tissue engineering is the reconstruction of skin (Howard et al. 2008).

24.7.7.1 Artificial Skin

Human skin tissue reconstitution is very important for skin grafting, and the artificial skin may be constructed from two layers obtained from human cells cultivated in vitro. The dermal layer equivalent can be designed from fibroblasts, and the epidermal equivalent is layered on the dermal surface. The dermal equivalent construct is developed by disassociating fibroblasts from a tissue followed by mixing it with the predetermined proportion of collagen in the culture medium. This is later poured into a mold of the specific geometry of the tissue construct, and the fibroblast-based condensation of collagen into fibrils forms a gel-like matrix that excludes surrounding fluids. The epidermal equivalent is developed from keratinocytes and is layered on the surface of the dermal equivalent. This living bi-layered skin of keratinocytes and fibroblasts is derived from neonatal foreskin tissues that lack antigen presentation (Brohem et al. 2011).

24.7.8 Cell Therapy

The hematopoietic stem cells (HSCs) can expand and undergo differentiation into other types of cells. The reason behind the high turnover of HSCs is their continuous growth and replacement. The ex vivo proliferation can be manipulated by careful

culture control of stem cells, and the HSCs can be an appropriate target for somatic cell gene therapy (Fuchs 2012).

24.7.9 Drug Screening and Toxicity Tests

The potential toxicity and effectiveness of newly discovered therapeutic molecules have been tested on whole animal models for many years, and there are some disadvantages and objections associated with these tests (high in cost and moral concerns for the animals). Mammalian cell culture systems can offer an alternative test system to overcome these disadvantages. Using cells for toxicity tests provides more efficient and rapid screening of novel compounds. The test system can be designed to evaluate various effects like a breakdown of membrane permeability, tissue-specific response, simulated wound healing, genetic effects, etc. (Astashkina et al. 2012).

24.8 Conclusion

Animal cell culture technology involves the culture of animal cells in artificial media. In this technique, cells are removed from tissue or whole organism and are cultured in artificially controlled conditions. The in vitro culture of the animal cell requires specialized techniques and types of equipment. The cultured cells could be anchorage dependent or anchorage independent (suspension cell cultures). When cells are directly cultured after removing from parent tissue or organisms, then such cultures are called primary cultures. When primary cultures are first subcultured, then they are known as secondary culture. After many subcultures when a single cell type is maintained, it is called a cell line. There are many cell lines which are maintained in animal cell culture laboratory and are used in research and development, e.g., HeLa cells (human cervical cancer cells), Chinese hamster ovary cells, Jurkat cells, and Sf9 cells. In hybridoma technology, immortalized cell lines secreting specific antibodies are produced and are known as monoclonal antibodies.

For culturing animal cells in vitro, a specialized culture media is required which must contain essential nutrients and growth factors needed for cell growth, survival, and proliferation. Cell culture media could be natural or synthetic. Natural media are generally biological fluids such as plasma, serum, or embryo extracts, and synthetic media are artificially prepared by mixing growth factors and suitable organic and inorganic compounds.

Laboratory design is equally important for in vitro culture as animal cells are very prone to microbial contamination. It must minimize contamination and provide an aseptic environment. Some essential set of equipment are also required in animal cell culture laboratories. Biosafety cabinets or laminar airflow cabinets are fitted with HEPA filters and provide a sterile environment for operation with samples and are also helpful in protecting operators. The incubator controls temperature, CO_2 content, and relative humidity necessary for the growth of cells. Some other important

equipment/instruments are culture vessels, microscopes, centrifuges, and liquid nitrogen storage.

Animal cell culture technology shows great applications in research and development especially in the field of biomedical research. This technology is involved in gene therapy to treat many genetic disorders. Animal cell culture technology is also implied in the production of different biologicals such as viral vaccines, interferons, therapeutic proteins, growth factors, hormones, biopesticides, and monoclonal antibodies. Nowadays animal cell culture technology is also used for plastic surgeries and drug screening and toxicity testing. The wide acceptance of cell-based assays for screening new drugs has minimized animal sacrifices to a great extent. Without animal cell culture technology, scientific revolution cannot be considered.

Declaration The authors declare no conflict of interest.

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P. Verma (ed.), *Industrial Microbiology and Biotechnology*,
https://doi.org/10.1007/978-981-16-5214-1